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EFFECTS OF A SERIES OF STEROIDS ON OVULATION AND REPRODUCTION IN PIGEONS

(One plate)

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UNLIKE fishes, amphibians, and mammals, birds have shown little ability to ovulate as a result of hormone administration. In all classes of vertebrates the cause of ovulation remains a moot question. The literature to 1938 was reviewed by Hartman (1939), and conditions attending normal and mechanically induced ovulation in the fowl were studied effectively by Phillips and Warren (1937). It is known that at least one steroid (an estrogen) is present in birds in unusual amount for many hours before and after ovulation and that this estrogen then greatly increases serum calcium and fat and probably stimulates the adrenal cortex and parathyroids. Probably, however, no momentary or quite temporary change in blood content of estrogen is the actual cause of ovulation. The studies of Phillips and Warren, nevertheless, make it probable that conditions which result in prolonged tension in the muscle fibers of the follicular wall of a mature follicle will promote ovulation in birds. Witschi (1935, 1936) obtained normal ovulation in a few house sparrows and weaver finches injected with serum from pregnant mares. Many failures of normal ovulation in bird ovaries stimulated by pituitary gonadotrophin could be cited.

At the outset of this study it was thought that an understanding of the humoral basis of ovulation would be advanced by a determination of the possibly quite unlike effects of steroids of the various types—estrogens, androgens, progesterone, and desoxycorticosterone. The qualitative results on this point, however, show perhaps more of similarity than of difference. Our initial tests, nevertheless, provided a new item of interest to this general problem when they made it evident that an unusual type of ovulation or of atresia results from the administration of all or most of the steroids studied. That process could be studied advantageously in this material. Again, unexpected effects of some of these steroids on oviposition and on the formation of eggshell also became apparent at the outset of the investigation. The present study was therefore directed to the gross effects of a series of steroids on ovulation, false ovulation, oviposition, and shell formation in ring doves and pigeons.

MATERIAL AND METHODS

Ring doves and pigeons offer the definite advantage that in them the hours at which both ovulation and egg-laying occur is fixed and known. The females of these species lay (normally) a clutch of two eggs; the first of the pair is laid by doves at about 5:00 P.M. (4:00 P.M., pigeons) of a given day, and the second at about 9:00 A.M. (3:00 P.M. for pigeons) of the second day thereafter. The ovulation of any ovum occurs in doves about 39 hours (pigeons, 43 hours) before it is laid as a completed egg, and its final 25 hours is spent in the shell gland, where it receives its shell. The interval between the ovulation of the "first" and "second" eggs in doves is about 41 hours (pigeons, 47 hours). The "first" egg can usually be palpated in the oviduct at least 33 hours before it is to be laid. Thus, by 9:00 A.M. of the day before this egg is to be laid (the shell is then still soft or absent) it is a simple matter to detect its presence in the oviduct.

Ovulation of the second ovum of a clutch usually occurs in doves about 2 hours (pigeons, 4 or 5 hours) after the "first" egg has been laid, i.e., at 6:30–8:00 P.M. When oviposition of a "first" egg is delayed (retained in the shell gland) beyond the hour at which the "second" egg should break from the ovary, this break may be prevented; and, if the break actually occurs, the yolk more often falls into the body cavity than into the oviduct.

In untreated birds a clutch occasionally consists of a single egg laid. In these cases one of three things may have occurred: (*a*) only one egg matured; (*b*) a second ovum ovulated into the body cavity; (*c*) some adverse agency caused regression or atresia of one or the other ovum. Nevertheless, when a hen (dove or pigeon) is in good condition and has regularly laid normal clutches of two eggs, it may be assumed that nearly all failures of that female to lay two eggs in a clutch after the institution of experimental treatment are traceable to the experimental procedure used.

Mature mated female birds, mostly ring doves, were used in this study. A few were treated just before the first egg in life was to be laid, but most of them had a longer record for egg production which indicated that they regularly laid clutches of two eggs. The foregoing brief description of the laying cycle serves to explain why it is easy to perform closely timed experiments on ovulation and on oviposition in these birds. Thus, if one wishes to inject a substance at any specified number of hours before an expected ovulation, this is easily done in the case of the ovulation of the "second" ovum, provided one is certain of the stage of the "first" egg in the oviduct; such certainty is attained by a little experience in palpation of the eggs. In all cases described here we refer to ovulation of a "second" ovum. For example, many of these tests were made on birds injected at 9:00–10:00 A.M. of the day when the soft-shelled "first" egg could first be palpated. For doves this was about 32 hours (pigeons, 31 hours) before time for this egg to be laid, and 34 hours (doves) or 35 hours (pigeons) before the "second" ovum would break from the ovary. Other birds were injected about 24 hours later, thus limiting to 6–10 hours the time during which the injected steroid would have opportunity to act prior to normal oviposition of "first" eggs and to normal ovulation of the "second" ovum.

Several crystalline steroid[1] substances were utilized; desoxycorticosterone acetate, progesterone, dehydroandrosterone, and estradiol benzoate were most used, and only a very few tests were made with androsterone and estrone. In general, these substances

[1] These hormones were kindly supplied by Dr. Erwin Schwenk, Schering Corporation, Bloomfield, New Jersey.

were dissolved in sesame oil and injected into the breast muscles; propylene glycol was used as a solvent for progesterone in a few instances. In three cases as much as 0.6 cc. of oil was required to administer the desired quantity of estrone; otherwise, never more than 0.3 cc. of solvent was used. One series of birds was injected with 0.3 cc. and another with 0.6 cc. of sesame oil only. In all cases the total dose of the hormone (or oil) was given at a single injection.

EXPERIMENTAL

Desoxycorticosterone acetate.—Effects of desoxycorticosterone acetate on ovulation, oviposition, and eggshell formation were observed on forty-three ring doves at dose levels of from 2.25 mg. to as low as 0.05 mg. The injections were made from 5 to 36 hours before ovulation time, or from about 3 to 32 hours before time for the "first" egg to be laid. These tests are summarized in Table 1. The results of our preliminary test—made on four common pigeons—are included in this tabulation. When these four hens presumably had an ovum (second of the clutch) ready to ovulate 8–10 hours (two tests) or 30 hours (two tests) later, they were each injected with 6.0 mg. of desoxycorticosterone acetate in sesame oil. In these treated birds there was delay in the laying of both of the two "first" eggs, which were longest exposed to the treatment; and, since in no case was a "second" egg laid, apparently there was also a disturbance of ovulation of all of the four "second" eggs. One column of Table 1 includes all cases (thirty-one doves) examined on the day after ovulation should have occurred and which should have had a "second" egg in the oviduct but did not. In a few cases autopsy was delayed for 1 or 2 additional days, but the findings were apparently not affected by this delay. Two associated columns record two types of change in the follicles and yolks of "second" ova.

Autopsies of several birds at the expected hour of ovulation and at various periods thereafter provided a fairly clear picture of the gross effects of the treatment on the follicle and yolk of the "second" ovum. A group of five birds which received the highest dosage (2.25 mg.) 26 hours before ovulation time were killed close to the time of ovulation (5:45–8:00 P.M.). Intact normal "second" follicles were observed in three cases; in the two others much yolk remained in its normal position, some yolk was present in bulges on the wall of the follicle, and some yolk had escaped into the body cavity. Within 1 hour of the time for ovulation the unovulated follicles appeared wholly normal, and in one case the fimbria had not clasped the follicle at 7:30 P.M., though it had done so in another case at 5:45 P.M. Six birds which had received the maximum dose (2.25 mg.) at 26 hours before the expected hour of ovulation were sacrificed when this was 24–72 hours overdue; in all cases we found an empty follicle (which had released the "first" egg) and an injured (atretic) follicle of the "second" egg, whose appearance varied somewhat according to lapse of time since treatment. Each of these six "second" ova had been ovulated in part into the walls of the follicle itself and in part into the body cavity. Normal ovulation was not obtained when maximum dosage was given 26 hours before the time for its occurrence, though six such cases did occur when treatment preceded this event by only 5 hours.

Several of the follicles concerned in the false or abortive ovulations noted above have been sectioned serially and studied histologically. This study leads to the conclusion that the granulosa and theca interna of the follicular wall are first broken at one or more points and that considerable quantities of yolk pass into the looser stroma of the theca interna, which gives the follicle an irregular, lumpy appearance. Later, in the majority of cases

TABLE 1

SUMMARY OF TESTS WITH DESOXYCORTICOSTERONE ACETATE AND PROGESTERONE IN RING DOVES

Dosage		Number									
			Laid				Killed in Study of Faulty Ovulation			"First" Eggs Studied for Weight Loss	Average Weight Loss per 24 Hours (Gm.)
Milligrams	Hours before Second Ovulation	Cases	Two Eggs	Only One Egg	Oviposition of "First" Egg Delayed	Killed near Ovulation Time	Cases	Atretic Follicles Found	Yolk in Body Cavity		
		Desoxycorticosterone Acetate									
2.25	26	11	0*	11*	1	5*	6*	8	5	8	1.60
2.25	5	10	6	4	0	0	10	3	2	10	0.07
1.10	26	2	0	2	0	0	2	2	2	1	0.80+
0.50	26	2	0	2	1	0	2	2	2	2	1.27+
0.25	26	4	1†	1	1	2	2	1	1	3	0.25
0.10	26	5	0	5	0	1	4	5	4	5	0.42
0.10	11	1	0	1	0	0	1	1	1	1	0.07
0.05	36	5	3	2	1	0	1	0	0	5	0.22
0.05	26	1	0	1	0	0	1	1	1	0	.....
Pellet	26	2	0	2	2	0	2	2	2	2	1.10
Total	.....	43	10*	31*	6	8*	31*	25	20	37	.....
6.00‡	30	2	0	2	2	.....	.....	.....	.....	.....	.....
6.00‡	8	2	0	2	0	.....	.....	.....	.....	.....	.....
0.30‡	26	1	0	1	0	0	1	1	1	.....	.....
		Progesterone									
0.50	26	3	0	3	1	1	2	2	2	2	1.55
0.50	10	1	1	0	1	0	0	0	0	1	0.05
0.30	29	1	0	1	0	0	1	1	1	1	0.19
0.10	26	3	0	3	1	0	2	2	2	3	0.11
0.10	10	3	1	2	1	0	2	1	1	2	0.07
0.05	26	6	1	5	0	0	2	2?	0?	5	0.09
0.30‡	26	3	0	3	1	0	3	3	3	3	0.65
Total	.....	20	3	17	5	1	12	11	9	17	.....

* Number of two-egg and one-egg clutches is here possibly affected by those "killed near ovulation"; these latter, in turn, affect the number of "cases of faulty ovulation."

† Both eggs laid within a period of 25 hours, the "first" delayed.

‡ Tests made on common pigeons.

the theca externa ruptures and permits yolk to escape into the body cavity. Figure 1 of Plate I shows the wall of a normal follicle; Figures 2 and 3 show the break in the granulosa and theca interna which permits yolk to enter the looser stroma of the externa; Figure 4 shows the minute break found in the outermost membrane of a follicle which was permitting yolk to escape into the body cavity.

This account requires mention of the fact that in several cases the follicle of the "first" egg, which had ovulated before the treatment, did not atrophy in a normal fashion following administration of desoxycorticosterone. Degeneration of the walls seemed less rapid, and their outer surfaces sometimes developed a lumpy appearance, resembling that observed in the follicles of "second" eggs.

In two birds a pellet of this substance was implanted subcutaneously 26 hours before ovulation. This method of administration proved (Table 1) highly effective.

The column at the extreme right in Table 1 gives the average loss of weight (loss of water per 24 hours at 103° F.) for "first" eggs whose shells were produced during the action of the hormone. A high rate of loss indicates a thin (or porous) shell. Larger doses and longer periods of action of this substance plainly produced a thinning of the shells. The average loss of weight for normal eggs is sufficiently indicated by the data of Table 2. Other definitely significant effects of desoxycorticosterone were not noted. In a small number of cases (six out of forty-three) the oviposition of the "first" egg seemed to be definitely delayed by this substance, but an almost equal number of instances of premature egg-laying attended its use. Desoxycorticosterone thus seems to have an adverse effect on the co-ordination of the muscular activities of the oviduct, in addition to an inhibiting action upon the secretion of eggshell.

Progesterone.—Effects of progesterone were observed in twenty tests—seventeen ring doves and three common pigeons. The data supplied in Table 1 show results very similar to those obtained with desoxycorticosterone acetate. One dove, which received 0.1 mg. at 10 hours before ovulation, had a quite normal follicle when killed at 20 hours after ovulation time. In two of the three treated pigeons more than one atretic follicle was found at killing, and relatively large quantities of yolk had escaped from the atretic follicles into the coelom in all these three common pigeons.

It is clear that all dosages (0.05–0.5 mg.) of progesterone used in these tests tend to prevent normal ovulation; only three of an expected twenty of the latter were obtained. Follicles, at least after a delay, lose more or less yolk to the body cavity, and spaces within the follicular walls become filled with yolk. There is some tendency to delay oviposition of eggs already in the oviduct; and, when administered at or before the beginning of this process, doses of 0.3 and 0.5 mg. inhibit the laying-down of eggshell.

Controls.—It is convenient to present data for control tests in Table 2. As much as 0.6 cc. of sesame oil was required to dissolve the largest quantity of estrone (2.0 mg.) used, but otherwise 0.3 cc. was the maximum amount of oil used as a solvent. Sesame oil was administered to a series of eighteen doves, and propylene glycol (used in part as solvent for progesterone) was used in four tests. These solvents, with one or two possible exceptions, when 0.6 cc. of oil was used, were without notable effect.

Dehydroandrosterone.—Most injections of dehydroandrosterone were made at 30–34 hours before ovulation. The results are summarized in Table 2. It was not desirable to kill many of these females which failed to ovulate a "second" egg after dosage with dehydroandrosterone (and estradiol benzoate); but a few autopsies show that here, also, certain failures to ovulate could be traced to atresia of the follicle. It may be assumed that

TABLE 2

SUMMARY OF TESTS WITH SESAME-OIL CONTROL, ESTRADIOL BENZOATE, ESTRONE DEHYDROANDROSTERONE, AND ANDROSTERONE IN RING DOVES

Dosage		Number								
			Laid						"First" Eggs Studied for Weight Loss	Average Weight Loss per 24 Hours (Gm.)
Milligrams	Hours before Ovulation Time	Cases	Two Eggs	Only One Egg	Oviposition of "First" Egg Delayed	Killed for Study of Delayed Ovulation	Laid "Second" Egg on Time	"Second" Egg Laid One Day Late		
		Sesame-Oil Control								
0.6 cc.....	26	8	6	2*	0	2*	6	0*	6	0.074
0.3 cc.....	24–26	10	9	1	0	0	9	0	3	0.063
Total..	.....	18	15	3*	0	2*	15	0*	9	.....
		Propylene Glycol Control								
0.2 cc.....	26	4	4	0	0	0	4	0	2	0.057
		Estradiol Benzoate								
1.0.....	26–34	25	7	18	4	3	5	2	2	0.078
0.5.....	26–34	78	49	29	8	1	25	24	6	0.067
Total..	.....	103	56	47	12	4	30	26	8	.....
		Estrone								
2.0.....	26	3	1	2†	0	2	1	?†	3	0.09
0.75.....	26	9	5	3†	0	1	5	?†	5	0.065
Total..	.....	12	6	5†	0	3	6	?†	8	.....
		Dehydroandrosterone								
4.0.....	32	1	0	1	1	1	0	0	.....	.....
3.0.....	28–31	8	3	5	0	3	3	0	2	1.70
2.0.....	29–34	33	24	9	0	1	23	0	2	0.187
Total..	.....	42	27	15	1	5	26	0	4	.....

* One with normal follicle but ovulation 20 hours overdue; other with atretic follicle and a trace of yolk in body cavity.

† Two birds on 2.0-mg. and one on 0.75-mg. dosage killed at 17 hours after time for ovulation of the "second" egg; thus these three cases are partial tests only (ovulation after 24 hours was possible, but not tested).

TABLE 2—*Continued*

Dosage		Number							"First" Eggs Studied for Weight Loss	Average Weight Loss per 24 Hours (Gm.)
Milligrams	Hours before Ovulation Time	Cases	Laid		Oviposition of "First" Egg Delayed	Killed for Study of Delayed Ovulation	Laid "Second" Egg on Time	"Second" Egg Laid One Day Late		
			Two Eggs	Only One Egg						
		Androsterone								
2.0.....	26	4	0	4	2	4‡	0	?‡	4	0.093
1.0.....	26	1	0	1	0	0	0	0	.....	.....
0.5.....	26	1	1	0	0	0	1	0	1	0.078
Total..	.....	6	1	5	2	4‡	1	?‡	5	.....

‡ Two normal follicles and two with lumps of yolk in theca externa at 17–43 hours past time of ovulation.

failure to lay the "second" egg in most (if not all) of the other cases had a similar basis. A dose of 2.0 mg. permitted twenty-four out of thirty-three birds to ovulate and lay a "second" egg, and this shows that on the basis of weight this substance is relatively much less effective in preventing normal ovulation and producing follicular atresia than is either progesterone or desoxycorticosterone acetate. "First" eggs from birds given dehydroandrosterone had inadequate shells.

Estradiol benzoate.—The action of this substance on ovulation was peculiar in one respect, which should be described at this point. The first bird of this series of one hundred and three tests received 1.0 mg. of estradiol benzoate 26 hours before ovulation time. Although the "first" egg was laid a little early, no "second" egg was palpable in the oviduct at 17 hours after it should have ovulated, and the bird was then killed. A quite normal intact follicle was found. This experience led us to delay killing for a longer interval in all later cases in order to see whether such uninjured follicles might ovulate at or close to 24 hours of delay. Our results indicate that this amount of delay occurred almost as frequently as did normally timed ovulation (26:30) and about half as frequently (26:47) as permanently suppressed ovulations. The tabulated data on this point are in terms of oviposition, not of ovulation; but here the two items almost certainly coincide. In these cases egg-laying was delayed just 24 hours; and shell formation, fertility, hatchability, etc., were in no wise affected. In fact, numerous young were hatched and reared from such eggs. Such a delay as this almost never occurs spontaneously.

An occasional autopsy made about 1 day after time for an ovulation revealed a normal, instead of an atretic, follicle after desoxycorticosterone acetate (one case), progesterone (one case), and dehydroandrosterone (one case). Several birds which received those substances and did not ovulate on time were given full opportunity before autopsy to ovulate their "second" egg at 24 or 48 hours late. In no case did this occur, and all autopsies delayed for 2 or 3 days revealed an atretic follicle. It is thus observed that after desoxycorticosterone acetate, progesterone, or dehydroandrosterone an atresia may not always be evident at the normal ovulation time, but ovulation will not occur at all unless

at the normal time. In this respect our experience indicates that the action of estradiol benzoate differs from the above-named substances. It is probably true, however, that atresia occurs after estradiol in many cases when ovulation fails to occur at the normal hour or at 24 hours later. That this atresia is not always delayed for 24 hours or more beyond ovulation time was ascertained in two cases.

Untabulated observations show that twenty-one birds receiving estradiol benzoate laid the "first" egg 2–4 hours earlier than is usual; in only twelve cases was oviposition of this egg delayed by 2 or more hours. In a very few instances desoxycorticosterone and progesterone also apparently hastened the laying of "first" eggs by as much as 2 hours. The shells of the few "first" eggs studied for rate of weight loss were not perceptibly affected by treatment with estradiol benzoate.

From the data presented in Table 2 it appears that 1.0 mg. of estradiol benzoate interferes with 72 per cent of ovulations and that 0.5 mg. prevents 37 per cent. Two irregular cases are not included in the tabulation. One female received 0.5 mg. of estradiol benzoate at 34 hours pre-ovulation, and, though the "first" egg presumably was laid, it could not be found; another egg, supposedly the "second," was laid a few hours later (1:50–3:20 P.M.) than that egg would be expected. Another female received 0.5 mg. of estradiol benzoate 26 hours before ovulation time but failed to lay the "first" egg on time. When the "first" egg was 24 hours overdue, pitocin was given to expel it. Within 20 minutes both the "first" egg (with hard shell) and the "second" egg (with soft shell) were expelled from the oviduct. In this rather exceptional case a "second" ovum had ovulated normally, although a "first" egg was still in the oviduct.

In another study Riddle and Dunham (1942) have shown that some of the estradiol benzoate injected in these tests entered the yolks of "second" eggs and there brought about partial sex-reversal in genetic male embryos which hatched from these ova.

Estrone.—Results from twelve doves treated with estrone are recorded in Table 2. At a dosage of 2.0 mg. one bird laid the "second" egg normally, and the other two were killed on the day after ovulation time in order to discover why the "second" egg had not reached the oviduct. In both cases the follicle of the "second" egg was normal. Six of the nine birds receiving 0.75 mg. ovulated and laid the "second" egg normally. In one case a normal unovulated follicle was found at 17 hours after ovulation should have occurred. In the other two cases only one egg was laid, and no autopsy was performed, since both of these birds had previously laid single clutches and our tests were made especially to increase our tests on oviposition and thickness of shell of "first" eggs.

Estrone showed no tendency to affect the time of oviposition or the amount of shell formed on "first" eggs. It may be noted here that Ökland (1936) fed tablets of estrogen to laying hens in a study of possible effects on the sex ratio. No effect on either sex or a reproductive process was noted, but the hormone was probably inactivated before absorption.

Androsterone.—Only six tests were made of effects of androsterone (Table 2). The single bird tested at lowest dosage (0.5 mg.) laid both eggs normally; the single bird given 1.0 mg. laid no "second" egg (atresia probable), but there was no autopsy. Four birds received 2.0 mg., and all failed to ovulate. Two of these, however, also failed to lay the "first" egg; in one of these the follicle was normal 17 hours after it should have ovulated, and in the other (43 hours overdue) there was atresia of the "second" follicle, together with some yolk in the body cavity. The remaining two cases laid their "first" eggs on time but were killed at 17 hours after the time when the "second" eggs should have ovu-

lated; here one "second" follicle had become atretic and the other had not. These tests do not decide whether ovulations delayed by androsterone tend to occur after a 24-hour delay, as they do after estradiol benzoate. Schoeller and Gehrke (1933) observed in fowl that daily injections of 0.5 capon unit of noncrystalline testosterone (Colu) or 5–10 rabbit units of impure progesterone (Proviron) usually stopped egg-laying, although (confirming Noether) 1,000 mouse units of estrone (Progynon) was without effect.

Shells of "first" eggs were little, if at all, affected by the treatment with androsterone. Failure to lay the "first" egg on time in two out of six tests suggests, but does not prove, that this was a result of the treatment.

DISCUSSION

Desoxycorticosterone acetate and progesterone have here been shown to have a similar inhibiting action on ovulation, in delaying oviposition, and in diminishing eggshell formation in ring doves and in pigeons. Others have demonstrated a life-maintaining action of desoxycorticosterone acetate in adrenalectomized animals which Gaunt and Hays (1938) likewise obtained with progesterone in adrenalectomized ferrets. Cantarow and Rakoff (1940) have shown that these two substances exert a similar effect in increasing the rate of entrance of sodium and chloride into 5.5 per cent dextrose solution introduced into the peritoneal cavity. The literature contains reports of still other similarities of action of these two substances. All steroids used in this study are capable of preventing ovulation, but desoxycorticosterone acetate and progesterone are exceptionally potent. These substances (given 26 hours before ovulation time) are usually effective in doses as low as 0.05 mg., but the effective dose of the other substances seems to be several times higher. Thus ovulation occurred in forty-nine out of seventy-eight tests (twenty-four were delayed for 1 day) with 0.50 mg. of estradiol benzoate, and in twenty-four out of thirty-three tests with 2.0 mg. of dehydroandrosterone. Inadequate data for estrone and androsterone indicate their lesser ability to delay ovulation. Shapiro (1936) and Zwarenstein (1937) have reported the induction of ovulation in the South African clawed toad (*Xenopus laevis*) by means of progesterone as well as with other steroids. Langan (1941) has reported similar results in the frog (*Rana pipiens*). We obtain a dissimilar result in birds.

Desoxycorticosterone and (particularly) progesterone tend to delay oviposition. On this point their action seems to differ from that of estrone and dehydroandrosterone. In their ability to diminish the deposit of eggshell, desoxycorticosterone and progesterone are similar to dehydroandrosterone and unlike estradiol, estrone, and androsterone. It should be stated that the relative thickness of eggshells was observable grossly on many eggs whose rate of weight loss was not measured; several eggs were almost devoid of shell.

Studies on mammals have noted that progesterone there inhibits ovulation. Jares (1932) reported that the minimal dose of pregnancy urine (P.U.) necessary to induce ovulation in rabbits was definitely larger when the ovaries contained active corpora lutea. Makepeace *et al.* (1937) injected postpartum rabbits with progestin or progesterone for 5 days before attempting to mate them. Most does then refused to accept the buck, and ovulation did not occur in those which did copulate. Some ovulations, however, were obtained in such does by P.U. injections, and this was regarded as an indication that the progesterone injections had suppressed ovulation indirectly through action at a point more central in the ovulation-provoking mechanism.

Dempsey (1937) found that progesterone suppressed ovulation (with regression of fol-

licles) in the guinea pig, although it did not interfere with growth of follicles up to the size attained 2 or 3 days before ovulation. He regarded this as an indication that progesterone does not interfere with follicle-stimulating hormone production but does suppress luteinizing hormone output; the follicles might thus grow to nearly mature size but do not ovulate because of the lack of luteinizing hormone. Riddle and Lahr (unpublished data) find evidence, however, that progesterone and desoxycorticosterone given at a 2.0-mg. dose level over 10 days markedly reduce the size of the testes in adult male ring doves and, further, that testosterone propionate has no effect, while crystalline androsterone (not Erugon) increases testis size in such adults. This result could be interpreted as an indication that the two first-named substances (but not the last-named) suppress follicle-stimulating rather than the luteinizing hormone output. It is notable, however, that in the investigations of Dempsey and of Riddle and Lahr dosage was continued over a period of either 20 or 10 days, while in the present study only one injection was given. Interference with ovulation by the several substances employed by us are not adequately interpreted as indirect results of suppression of the output by the pituitary of a substance necessary for ovulation, though the twenty-four cases of ovulation delayed for 24 hours by estradiol benzoate would seem in good accord with that view. Again, other studies of this laboratory have probably uniformly failed to induce ovulation in fowl (Bates, Lahr, and Riddle, 1935) and doves by partly purified pituitary extracts which produced much ovarian and follicular growth; these extracts must have contained appreciable amounts of both follicle-stimulating and luteinizing hormones. The simultaneous administration to birds of mammalian ovulation-inducing substances and desoxycorticosterone acetate or progesterone should provide informative results.

Nothing observed in this study eliminates the possibility that a direct effect, or even a general systemic effect, is responsible for the "abortive" ovulations described here. In this connection it is useful to recall that each of these steroids promotes certain useful activities of the body; when, as in the present tests, they are introduced in excess, they disturb a series of processes which are probably under nicely balanced humoral control—processes as different, however, as ovulation, oviposition, and shell formation.

The form of follicular atresia or false ovulation encountered in this study seems to have been little identified and investigated. Wynne-Edwards (1939) figured a case found in a fulmar during a nonbreeding year. These birds in Labrador appear to breed not every year but intermittently. Davis (1941) seems to have made a special study of rather similar conditions in *Crotophaga ani* and *Molothrus bonariensis*, but only an abstract of his results has been published. Rupture of egg membrane, granulosa, and theca, permitting yolk to flow into the stroma of the ovary, was described; but no mention is made of a later loss of yolk to the body cavity. It is not clear whether this form of atresia was observed in eggs that should have ovulated normally or whether they were restricted to eggs remaining in the ovary after a normal number of eggs were laid. Much experience leads us to conclude that this type is not readily recognized apart from full information concerning reproductive status and history. Since the series of steroids employed in this study have rather generally produced this form of atresia, it seems probable that one or another steroid—perhaps from the adrenal cortex—is causally related to its normal occurrence in birds.

From observations in the fowl Phillips and Warren conclude that "a last minute deposition of yolk was not responsible for follicular rupture." The present data strongly support that conclusion. The twenty-four cases of ovulation delayed for (close to) 24 hours, with normal ovulation at that time, speak at once against a simple "pressure-to-burst-

ing" theory and for the significance of a diurnal state or condition which in doves is reached regularly near midnight in the case of a first yolk of a pair and 4–6 hours earlier (6:30–8:00 P.M.) nearly 2 days thereafter for the second yolk of the pair. The ovulation of the "first" egg appears to mark a sharp discontinuance of estrogen production in the follicle from which it escaped, and this perhaps forces the second ovulation to occur when there is less estrogen (from only one growing follicle) in the blood stream than at the first ovulation; it could therefore be supposed that the relative concentration of estrogen does not determine the moment of ovulation for any egg, but it may nevertheless share in those conditions which decide that "first" eggs ovulate at midnight and "second" eggs at 7:00 P.M.

It is found, as might have been anticipated, that a slight or moderate excess of either of the two estrogens tested has no adverse effect on shell formation. A failure to disturb oviposition applies to estrone but not to estradiol benzoate. In this case the difference concerns a function in which smooth muscle is involved, as does the bursting of the follicle; and it is possible that the estrogen (unidentified) produced by the bird exerts neither of these adverse actions.

SUMMARY

The action of a series of steroids on ovulation, oviposition, and eggshell formation has been studied in ring doves and pigeons. Treatment was by single intramuscular injection at 5–36 hours before the known hour for ovulation of the second egg of a clutch and while the earlier egg of the pair was in the bird's oviduct.

Desoxycorticosterone acetate, progesterone, dehydroandrosterone, estradiol benzoate, estrone, and androsterone all delay ovulation and result in abortive ovulation or atresia if sufficiently high dosage is used. Desoxycorticosterone acetate and progesterone, however, are highly effective at a dosage of 0.10 mg. and 0.05 mg., while the other substances were not always effective at dose levels ranging from 0.5 mg. to 2.0 mg.

Abortive ovulation or atresia of the type induced by these hormones is probably not uncommon among birds generally, but it has been little noted and described. Four figures of this condition are presented. At, or some hours after, the normal hour of ovulation the granulosa and theca interna of the follicular wall, together with the egg membrane, are ruptured at one or more points, from which more or less yolk flows into the loosened stroma of the follicle. In most cases the theca externa later ruptures and permits yolk to escape into the body cavity.

Desoxycorticosterone acetate, progesterone, and higher doses of dehydroandrosterone, unlike the other hormones tested, have an inhibitory action on the process of shell formation. Progesterone, desoxycorticosterone, and estradiol benzoate frequently delay the expulsion of the egg from the oviduct.

Estradiol benzoate was peculiar in that it rather frequently caused a 24-hour delay without other abnormality of ovulation. The interference with ovulation exercised by the other steroids used was of an "all-or-none" character.

The bearing of these results on certain theoretical questions is discussed.

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PLATE I

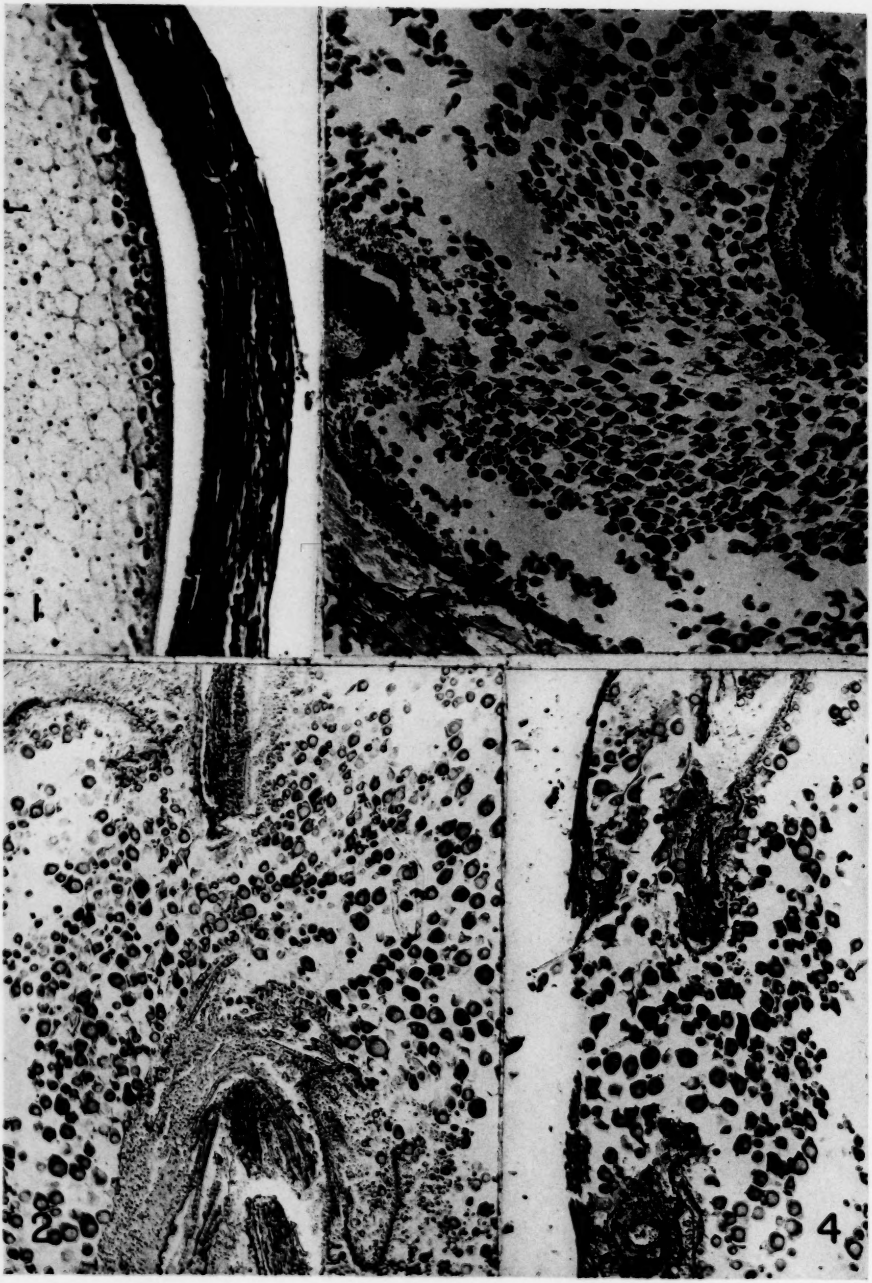

PLATE I

FIG. 1.—Section of a portion of a normal follicle showing (from left to right) yolk, vitelline membrane, granulosa, theca interna, theca externa (larger intercellular spaces), and epithelium (peritoneum). ×140.

FIG. 2.—Showing (after dose of 2.25 mg. of desoxycorticosterone acetate) the breakdown of the granulosa and theca interna at one point, permitting yolk to flow from its normal position (right) into stroma of the theca externa at left. ×70.

FIG. 3.—View of same follicle as foregoing at a wider point of the break, showing yolk material spilling into external layer of follicle wall (below). ×70.

FIG. 4.—Showing (after dose of 2.25 mg. of desoxycorticosterone acetate) break of epithelium of follicle wall (at left), permitting yolk to flow into body cavity from pockets of yolk formed in stroma of the theca externa of the follicle wall. ×70.

EXPERIMENTS ON DISTRIBUTION AND ACTIVATION OF THE NEPHROGENIC POTENCY IN THE EMBRYONIC MESENCHYME

(Six text figures and three plates)

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THE experiments to be discussed in this report are a continuation of an earlier investigation on the morphogenetic action of the Wolffian duct upon other parts of the embryonic urogenital tract in the chick embryo (1937). In that previous investigation the consequences of destruction or interruption of the Wolffian duct had been studied as produced by electrolytic damage to the growing end or further cranial levels of the duct. It had been found, in agreement with earlier results of Boyden (1927) and later experiments of Waddington (1938), that differentiation of the nephrogenic tissue into mesonephros took place only where that tissue was in contact with the Wolffian duct. Experimental embryos kept living up to the twelfth day of incubation showed a similar relation between ureteric bud and metanephric blastema. The latter failed completely to differentiate into nephrons if, after destruction of the Wolffian duct, the respective ureteric bud was missing. In its downward growth the Müllerian duct was found to follow the Wolffian duct and was therefore absent caudal to the level of destruction or interruption of the latter. Gonads and adrenal cortex developed normally in the absence of Wolffian duct and mesonephros. It was shown by comparing these results with the common malformations of the human urogenital tract that the same developmental relations prevail in the human embryo (1938); concerning the permanent kidney, this was further illustrated by the findings in an early stage of agenesis of the ureteric buds in a human embryo (1939). As to the Müllerian duct, a recent investigation of its relation to the Wolffian duct in human and chick embryos (1941*b*) has revealed that the topographical relations of the two ducts are so close at the time of the downward growth of the Müllerian duct that no inducing action of the Wolffian duct has to be assumed to account for the above-mentioned effect.

In the present series of experiments an attempt was made to test the nephrogenic power of mesenchyme other than that normally forming mesonephric or metanephric nephrons. Grafts were introduced in a place through which the Wolffian duct would normally have to pass during its growth toward the cloaca, and it was hoped that the duct would grow either through the graft or around it, thus being in contact with mesenchyme normally far away from the place of formation of urinary organs. In addition to results pertaining to this original question, new findings concerning the development of nephrogenic tissue in the absence of the Wolffian duct will also be described. The literature on these subjects was discussed in an earlier report (1937) and will therefore not be reviewed again.

MATERIAL AND METHODS

The experimental technique to be described here is adapted to experiments performed during the second day of incubation. Barred Rock eggs were used in the present series,

but no difference was noticed between these and eggs of unknown breeds used in previous experiments. At the desired time the eggs were removed from the incubator, cleaned with cotton soaked in 70 per cent alcohol, and placed in a wooden ring adapted to egg shape in the same position which they had occupied in the incubator. A strong splinter forceps was now used to make a small opening at the obtuse pole, opening the air chamber by perforating the shell and the attached layer of the shell membrane. By means of the same instrument a small drop of Ringer solution was now deposited at the highest point of the shell over the blastodisc, and through it another opening was made in the shell. If the shell membrane was perforated at the same time, the contents of the egg tended to sink down, displacing the air from the air chamber and aspirating the Ringer solution through the second opening. If this was not the case, the shell membrane was opened separately under Ringer solution. Two advantages are achieved by this procedure: the vitelline membrane over the blastoderm is moistened from the very moment of its exposure and does not tend to stick to the shell and the border of the opening, and the entire blastoderm sinks deeper without any egg white's having been removed. This is important because the blastoderm should never be allowed to come in contact with the border of the opening, or the window with which it will be sealed. Only in rare cases must a pipette be used to remove a small amount of egg white. Now the upper opening is widened, according to the position of the blastoderm. Size and position of the opening were not determined in advance because of the varying position of the blastoderm. A small curved forceps is now dipped into the egg white, and with it the exposed surface of the vitelline membrane is thoroughly moistened. Egg white keeps the surface moist much longer than does Ringer's solution. The same forceps is now used to cover the center of the blastoderm with an agar platelet soaked with neutral red. These platelets were prepared according to the methods described in the literature, kept in a 1 per cent solution of neutral red in 50 per cent alcohol, and rinsed in Ringer's solution before use. While the blastoderm is taking up the dye, the cellophane window may be prepared. It is cut so as to exceed the opening on all sides by about 2 mm., and a ring is drawn along its border consisting of a mixture of resin and beeswax (4:1). The mixture is kept melted in an evaporation dish for the duration of the experiments and applied with a heated spatula. The agar is now removed from the egg, and the vitelline membrane cut with a pair of Wecker's iris scissors. The instruments for the operation proper were made of blue glass wool. One of these glass hairs was fastened by the above-mentioned resin mixture to the end of a glass rod drawn out into a fine point. The hair was then bent as shown in Figure 1. In this position it was carried over a small gas flame and, when the proper curvature was reached, cut at the point indicated by an arrow in Figure 1, and the free part removed. This instrument was used for all cutting operations. Blue glass was used because colorless material would be almost invisible when immersed in the fluid covering the blastoderm. These instruments proved to be superior to those made by drawing out glass rods or by using hairs. All operations were performed under one of the commonly used binocular dissecting microscopes of the Greenough type. After the operation the blastoderm was again moistened with egg white as described above, and then the forceps containing some of the egg white between its ends was used to spread a thin layer of egg white over the inner surface of the prepared cellophane window inside the resin ring. This measure keeps moisture from condensing in the form of droplets and thus obstructing the view if the eggs are later removed from the incubator for observation. The cellophane was now placed on the egg in its correct position. Preliminary fas-

tening of the window was achieved by touching the resin ring from the side with the heated spatula. Some of the melted mixture was then taken from the dish with the same instrument and allowed to flow into the space between the border of the window and the egg shell for permanent sealing. The eggs were kept permanently in the same position, with the window at the top. It had been found, in agreement with Wolff's experience (1936), that this procedure yields better results than turning the eggs after the operation. The present experiments were not performed under aseptic conditions; the rate of survival was high enough to justify this procedure. In a laboratory equipped for sterile work it may be advisable to work aseptically; if, however, such equipment is not easily available, sterile work would involve additional working time and expenses which would by far exceed the amount of work and material otherwise lost by an occasional death from infection.

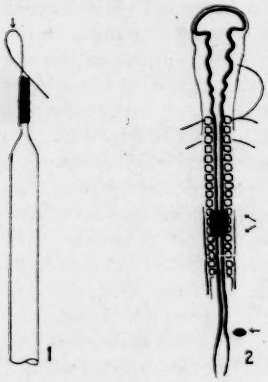


FIG. 1.—Glass instrument used in grafting experiments. The glass hair is cut at the point indicated by an arrow after it has been bent in a flame. Further explanation in the text.

FIG. 2.—Diagrammatic view of an 18-somite chick embryo, showing the operation performed in the present experiments. The part above the break represents the donor embryo; the part below shows the host. The original location of the tissues to be grafted is shown black (two arrows) in the upper part, and the site of the graft in the host is marked black (one arrow) in the lower part of the diagram.

The eggs were inspected twice daily, so that dead embryos could still be used for histological examination. All embryos were fixed in Bouin's solution, imbedded in paraffin, and cut serially at 10 μ. Azan stain was used routinely; it gives far better results than the usual haematoxylin or carmine stains, even in early blastoderms. Silver impregnation of the reticulum was used in several instances. This method was also employed to restain sections from the previous series of experiments for a study of the unstimulated metanephric blastema.

The particular operation carried out in the present series of experiments is shown in the diagrammatic Figure 2. Its upper part represents the donor, the lower part the host embryo. The rectangular area marked in black in the upper part of the figure, comprising all layers of the blastoderm, was cut out by means of the glass needle and a pair of Wecker's scissors. This included the neural tube, notochord, and the medial walls of the adjacent somites. Care was taken to include as little of the somites as possible, to make sure that the normal nephrogenic tissue situated laterally to the somites was not contained in the grafts. These pieces were transferred to host blastoderms of the same age and inserted at the level of the "segmental zone" in a transverse incision cutting through the lateral half of the prospective somite material and the adjoining area of the later intermediate cell mass. (Fig. 2). The incision perforated all layers of the blastoderm, and the graft was pushed into it regardless of its position. If it was pushed entirely through the incision and into the subgerminal cavity, another graft had to be prepared. At the stage of the operation, the growing end of the Wolffian duct of the host is found and can often be seen at the operation, slightly caudal to the last pair of somites. The graft was always placed at a level somewhat caudal to the end of the duct, so that it could be assumed that the immediate local reaction to the trauma had ended and healing

begun when the duct reached the graft. In only a few instances brain wall or an otic pit with the surrounding tissues was used instead of the grafts just described; there was no difference in the results of these experiments. All host embryos were operated upon on their right side; if the embryo dies spontaneously after the operation, the organs on this side are usually better preserved, because the blood vessels on the left side of the body are then tremendously dilated and congested with blood. In only a few instances the growing end of the Wolffian duct was destroyed by electrolysis, as had been done in the previously reported series. All embryos were fixed 2–5 days after the operation. Not all experiments and their results will be described here; the ones not mentioned showed conditions essentially similar to those presented in the following chapter.

OBSERVATIONS

THE WOLFFIAN DUCT IN ABNORMAL SURROUNDINGS

In most of the experiments pertaining to this group the Wolffian duct was found to have grown into or through the graft tissue. Only two cases were observed in which the duct had deviated from its direction of growth at the border of the graft—grown dorsolaterally through the body wall and opened on the body surface.

Embryo 709 was operated upon after 1 day and 22 hours of incubation at the 24-somite stage. The typical operation as described above was performed and the embryo was fixed alive at an age of 6 days and 21 hours, that is, about 5 days after the operation. In sections the organs of the left urogenital ridge appeared normal, as well as the cranial part of the right mesonephros. Only a dilatation of the Wolffian duct was found in that area. At the level of the graft (Fig. 7) the right Wolffian duct was found imbedded in the grafted tissues and could be traced caudally and, after bending dorsally, continued on cranially for a short distance. This curved part of the duct extended around a rod of cartilage of the graft. The mesonephros ended at the level at which the Wolffian duct left the host tissue. No induction of mesonephros or of any other differentiations was found near the duct within the mesenchyme of the graft. Caudal to the region of the experiment the mesonephros was absent, as in previous experiments of destruction of the Wolffian duct, with the exception of a small group of narrow tubules and a poorly developed glomerulus. The occurrence of such independent differentiations will be discussed later in this report.

Similar conditions were found in embryo 752, which had been operated upon after 1 day and 17 hours of incubation with 16 pairs of somites and fixed alive 3 days later. A short cranial portion of the right mesonephros was found on sections, attached to a considerably dilated Wolffian duct. The duct had entered the graft and ended there. Its dilated end was with its dorsal side closely applied to nervous tissue of the graft, and on the other sides it was surrounded by mesenchyme. No mesonephric differentiations were seen within the graft (Fig. 8). Farther caudally the mesonephros was again absent, with the exception of a small and irregular group of tubules similar to the one briefly mentioned in the description of embryo 709.

The two observations reported thus far indicate that mesenchyme of the graft does not respond to a stimulation by the Wolffian duct by differentiating into nephrons, as does the normal nephrogenic tissue. However, the possibility of the Wolffian duct itself being impaired in its stimulating capacity by the abnormal conditions of the experiment must be considered. There are, however, other experimental embryos which show that

the capacity of the Wolffian duct to induce differentiation of nephrons remains unimpaired by the experiments. In these cases the duct had succeeded in growing through the graft and again into the host's nephrogenic tissue, and there it showed again proof of its inducing power.

The first example of this group, embryo 725, had 12 pairs of somites after 1 day and 19 hours of incubation, when a piece of brain tissue was implanted at the usual site indicated in Figure 2. It was fixed after spontaneous death 3 days later. The right mesonephros had developed normally, in its cranial part, to the point where the Wolffian duct entered the graft. Within the graft the duct was surrounded by mesenchyme on all sides without producing any induction effect in that tissue (Fig. 9). Farther caudally, however, the duct again emerged from the graft and assumed its normal position in the urogenital ridge. In that area normal mesonephros was present, indicating a normal action of the Wolffian duct (Fig. 10).

A later stage of a similar condition was found in embryo 741. It had been operated upon after 1 day and 19 hours of incubation, in the 18-somite stage. Five days after the operation it was fixed alive. Serial sections revealed a normal left mesonephros; only the Wolffian duct was slightly dilated in its caudal part because of an incidental malformation of the cloaca (contact of the wall of the cloaca with the notochord), which apparently interfered slightly with the drainage of the duct. On the right side the mesonephros was normally developed cranial to the region of the operation. On examination of the sections in craniocaudal succession the graft appeared first medially, displacing the mesonephros dorsolaterally. At this level the mesonephros sharply decreased in size (Fig. 11) and disappeared completely at the level of the caudal part of the graft. In this caudal area the Wolffian duct was evidently completely surrounded by graft tissue (Fig. 12), whereas, farther cranially, part of the nephrogenic tissue seems to have been displaced with the duct and formed the small mass of mesonephros lateral to the graft. Caudal to the graft, the duct returned to its normal position and was again accompanied by a well-developed mesonephros (Fig. 13). The relations of the Wolffian ducts, mesonephroi, and the graft are shown in a graphic reconstruction, in which these structures appear projected on a frontal plane. The levels of Figures 11–13 are also indicated in this reconstruction (Fig. 3).

Another example of the ineffectiveness of the Wolffian duct in inducing nephric differentiations in the graft is embryo 724. It was operated on in the usual manner after 1 day and 19 hours of incubation, having developed 20 pairs of somites. Three days after the operation it was fixed after spontaneous death. The condition of its urinary organs is shown in a reconstruction (Fig. 4) prepared in the same way as that of embryo 741 and in photographs (Figs. 14–19). The organs of the left side appeared entirely normal. On the right side the first indication of a disturbance found upon craniocaudal examination of the series was a dilatation of the Wolffian duct, reaching a considerable degree and probably compressing the parenchyma of the mesonephros (Figs. 4, 14). A short distance cranial to the site of the graft a very narrow duct was given off by the distended Wolffian duct; it turned out to be the continuation of the Wolffian duct, whereas the wide part ended blindly in contact with the cranial surface of the graft. In Figure 15 both ducts are shown shortly after their separation. The distended part was connected with mesonephric tubules in its entire length; the narrow part, however, could be traced through the mesenchyme of the graft without any accompanying renal structures (Figs. 4, 16). Caudal to the graft, the duct again was dilated and received the

openings of a large group of mesonephric tubules situated dorsal to it in the host tissue (Figs. 17, 18). This second dilated part of the Wolffian duct was bent cranially and ended blindly near the caudal border of the graft (Fig. 4). By this atypically directed growth the duct reached an area caudal to the normal limits of normal mesonephros formation. The tubules attached to it in this region were situated far dorsally in a tissue

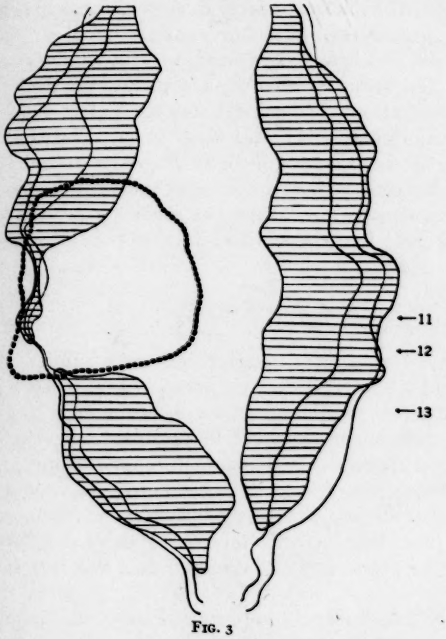


FIG. 3

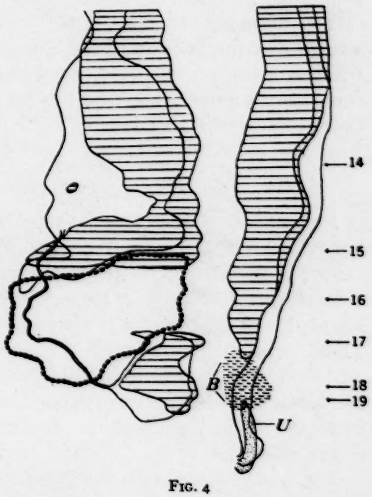


FIG. 4

FIG. 3.—Graphic reconstruction of the Wolffian ducts (thin outlines), mesonephroi (horizontal lines) and graft (heavy broken line) of embryo 741. These structures appear projected on a frontal plane. The arrows indicate the levels of the figures with corresponding numbers. Drawn at ×60; present magnification −27.

FIG. 4.—Graphic reconstruction of the urinary organs of embryo 724, shaded as Fig. 3. *B*, metanephric blastema; *U*, ureteric bud. Drawn at ×100; present magnification −27.

which normally forms tubules of the metanephros. Figures 4 and 19 show that the corresponding place on the normal left side of the embryo was occupied by the metanephric blastema containing the end of the early ureteric bud. This would indicate that the metanephric blastema on the right side of our embryo, being stimulated by the Wolffian duct instead of the ureteric bud, formed mesonephric tubules at a stage far prior to that in which it would normally form metanephric nephrons under the influence of the ureteric bud. The importance of this observation will be discussed in a later section.

It was mentioned above that in only two instances had the experiment caused the Wolffian duct to grow through the body wall and open on the dorsolateral surface of the

body. One of these is embryo 727, which had received a graft consisting of an otic pit and the surrounding tissues after 1 day and 20 hours of incubation, in the 17-somite stage. It was fixed alive 4 days after the operation. Figure 20 shows a section close to the cranial border of the graft; the mesonephros on the side of the operation had almost disappeared, and the Wolffian duct is cut longitudinally on its dorsolateral course toward the body surface. No mesonephros had differentiated caudal to the graft on the operated right side. It can clearly be seen that the duct is entirely devoid of mesonephric tubules on its course through the mesenchyme outside of the urogenital ridge.

The second case is not very conclusive. It concerns embryo 733, in which the region of the growing end of the Wolffian duct had been damaged by electrolysis at the 13-somite stage, after 1 day and 4 hours of incubation. The embryo was fixed alive 3 days after the operation. There, too, the Wolffian duct ended on the body surface (Fig. 5) in an area showing the effect of the operation by the presence of slight irregularities and an adhesion of the amnion to the epidermis. No tubules were found near the duct; but the tubules of the normal left mesonephros had already degenerated at this level, so that it could not be determined whether the abnormally located duct had induced tubule formation at an earlier stage.

DEVELOPMENT OF THE MESONEPHRIC BLASTEMA IN THE ABSENCE OF THE WOLFFIAN DUCT

Previous experiments (Gruenwald, 1937) had shown, in agreement with similar investigations of Boyden (1927) and Waddington (1938), that the mesonephric blastema shows, in the absence of the Wolffian duct, either no differentiation at all or only insignificant small cell cords. It was, therefore, quite surprising when independently developing nephrons appeared in the present series of experiments in a large number of embryos. These nephrons showed all transitions from small vestigial cell cords to conspicuous, well-developed groups of tubules and renal corpuscles. The occurrence of such structures has already been briefly mentioned in the preceding section. Several instances of differentiation of the mesonephric blastema in the absence of the Wolffian duct will now be described in detail.

The widest distribution of independently differentiating nephrons (Fig. 5) was found in embryo 733, described last in the preceding section. Besides cell cords (Fig. 23), as also seen in previous experiments, two groups of nephrons were found in this embryo, reaching a size similar to that of the normal left mesonephros in the same sections (Fig. 21). In addition, two poorly developed nephrons were seen in which highly deformed primordia of corpuscle and tubule could be distinguished (Fig. 22). All these areas were separated from one another and from the end of the Wolffian duct by regions in which no differentiation or only insignificant cords were present. The occurrence of external glomeruli on the left mesonephros, as indicated in Figure 5, will be briefly discussed below.

The largest single group of nephrons developing independent of the Wolffian duct was seen in embryo 773. On this embryo the usual grafting operation had been performed in the 11-somite stage, after 1 day and 19 hours of incubation, and it was fixed alive 3 days after the operation. The growth of the right Wolffian duct had stopped in this embryo at the cranial border of the graft. Close to the caudal border of the transplanted tissues a group of nephrons was found which on cross-section exceeded the size of the normal left mesonephros at any level of the same embryo (Fig. 24). The nephrons

were arranged within this group in such a manner that on the more cranial sections only the tubules, and on the following sections only glomeruli, were present. The location of this tissue in relation to the Wolffian duct and the graft is shown in a reconstruction in Figure 6. Its close proximity to nervous tissue of the graft should be noted here; it will be of interest in connection with the following observations. As can be seen in Figure 24, the embryo is developed somewhat asymmetrically; and, as a consequence, its left mesonephric ridge is situated in close proximity to the spinal cord and ganglia.

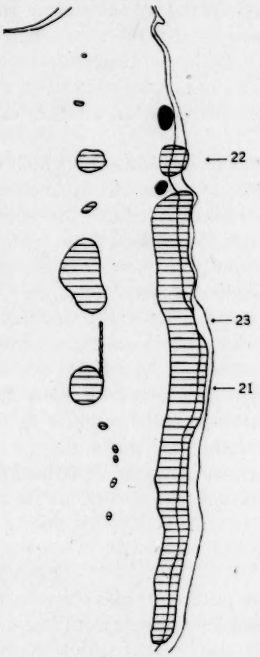


FIG. 5

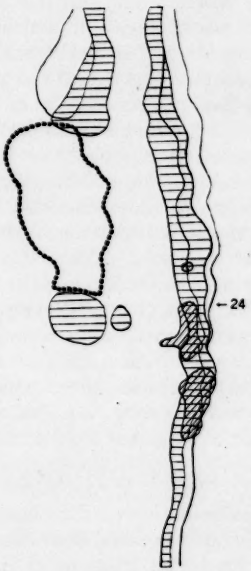


FIG. 6

FIG. 5.—Graphic reconstruction of the urinary organs of embryo 733, shaded as Fig. 3. Black: external glomeruli. Drawn at ×100; present magnification —27.

FIG. 6.—Graphic reconstruction of the urinary organs of embryo 773, shaded as in Fig. 3. Obliquely lined: nephrons probably induced by spinal ganglia. Drawn at ×100; present magnification —27.

Here groups of mesonephric tubules were found almost completely separated from the normal mesonephros and in close contact with the spinal ganglia. Figure 6 shows the distribution of these tubules. It is possible, although it cannot be definitely established, that these tubules have developed in the metanephric blastema, as in embryo 724. These observations, as well as the following findings, suggest that nervous tissue may stimulate the nephrogenic tissue in a similar way, as is normally done by the Wolffian duct.

Embryo 775 had received in the usual position a graft consisting of the otic pit and surrounding tissues after 1 day and 20 hours of incubation in the 20-somite stage. It was fixed alive 3 days after the operation. Serial sections revealed that the right Wolffian duct had been stopped by the graft and was, therefore, absent caudal to the region of the operation. The caudal end of the body showed a malformation which, as can be assumed almost with certainty, was not caused by the operation. It consisted of typical ourentery (Rabaud, 1900), characterized by growth of the caudal end of the body into the entodermal cavity instead of detachment in dorsal direction. The tail is then found in the cloaca or proximal part of the allantois and is covered by entoderm. In the present case the ventral flexure of the axial organs as caused by this malformation had resulted in bringing the nervous system into close proximity to the nephrogenic tissue not stimulated by the Wolffian duct. In this instance tubules are again present in close contact with nervous tissue (Fig. 25). Similar conditions were found in another embryo (766) with a malformation of its pelvic region.

Another peculiar observation was made in several of the embryos which had a defective mesonephros in consequence of the experiment. It was briefly mentioned in the description of the findings in embryo 733 that two external glomeruli were present on the surface of its left mesonephros. Similar glomeruli were found in five more embryos; and, as an example, one of two glomeruli seen in embryo 766 is shown in Figure 26. This embryo, which was mentioned briefly in the preceding paragraph, had been operated on in the typical manner in the 15-somite stage, after 1 day and 19 hours of incubation, and was fixed alive 3 days later. Its right Wolffian duct proved to have stopped growing when it reached the graft. At the level of its dilated end as well as at the level of the graft (Fig. 26), external glomeruli were present on the ventral surface of the otherwise normal left mesonephros. No attempt will be made to arrive at an explanation of this finding, because the cases available are not sufficient to allow an analysis of the conditions under which these glomeruli appear. Furthermore, normal controls will have to show whether or not these structures also occur in uninfluenced embryos. It is to be hoped that further experiments will clarify this point.

DEVELOPMENT OF THE METANEPHRIC BLASTEMA UNDER ABNORMAL CONDITIONS

Renewed examination of the behavior of the metanephric blastema in the absence of the ureteric bud appeared desirable after the discovery of new details concerning the mesonephric blastema. In many of the embryos of the present series the metanephric blastema of the right side was not stimulated by a ureteric bud in consequence of the interference with the growth of the Wolffian duct. The results were the same as those described in the previous report (1937). Although many embryos showed, in contrast to those of the earlier series, independent differentiation in the mesonephric blastema, none of this was observed in the region of the metanephros. The tissue in this area showed a denser structure for a long period but did not form any epithelial structures. New details of its minute structure were furnished by examining sections with silver impregnation of the angyrophil connective tissue. Sections from the new series of experiments, as well as restained specimens from previous experiments, showed that the uninfluenced blastema contains the same dense network of very delicate fibers as is present in normally developing kidneys in parts of the tissue not yet differentiating into nephrons. A comparison of both sides of embryo 745 (Fig. 27) may serve to illustrate this; similar histological resemblance was found in embryos of the twelfth day, although the unstimulated metanephric blastema is considerably reduced in size at this stage.

As mentioned above, there is strong reason to believe that in one of the experimental embryos (724) the metanephric blastema was stimulated by the Wolffian duct itself instead of the ureteric bud and, in response, produced mesonephric tissue. Unfortunately, this was an incidental finding, and no method could be devised up to the present time to produce this condition again. In another, far more problematic, case (773) it was suspected that the tissue stimulated by spinal ganglia to form mesonephric tubules may have been the metanephric blastema. Particularly the first-mentioned of these cases suggests that the nephrogenic blastema is potentially almost or completely uniform and that the inducing agent determines differentiation into either mesonephros or permanent kidney.

DISCUSSION AND CONCLUSIONS

Mesencyhme of the early chick embryo was tested for its ability to respond to inducing influences of the Wolffian duct by producing mesonephric structures. In contrast to the tissue normally forming nephrons, the mesenchyme surrounding the axial organs showed no sign of response when transplanted with these organs into the path of the Wolffian duct and thus eventually in intimate contact with the duct. The same was found to be true for the mesenchyme of the host's body wall, when it surrounded a duct diverted from its normal course by a graft. It can be concluded from these experiments that the ability to form kidney structures upon stimulation by the Wolffian duct either is limited entirely to the narrow area in which the mesonephros normally differentiates or exceeds it by very little. It was mentioned above and will be pointed out soon in more detail that the blastemas of mesonephros and metanephros are probably essentially alike in their reaction to inductors. If this should prove to be correct, the conclusions just reached would apply to the metanephric blastema and stimulation by the ureteric bud as well.

New and surprising results were obtained regarding the behavior of nephrogenic tissue in the absence of the inducing Wolffian duct or ureteric bud. In complete disagreement with previous results of the author and other investigators it was found that the mesonepheric blastema is capable of a considerable degree of self-differentiation. Excluding, for the present time, the instances in which stimulation by nervous tissue may be thought to be responsible for these differentiations, a considerable number of nephrons were found singly or in groups, showing all degrees of structural perfection from hardly recognizable cell cords to well-developed tubules with renal corpuscles. In all these cases, however, only a part of the unstimulated nephrogenic tissue had undergone self-differentiation. Of seventeen embyros of the present series containing mesonephric blastemas not reached by the Wolffian duct, ten showed differentiation. Only three of these belong to the group to be discussed later, in which nervous tissue may have supplied the inducing agent. In the remaining cases it might be suggested that the Wolffian duct had grown through the region of the operation, induced the differentiation in question, and then degenerated because of some damage suffered by the operation, or in growing through the graft. This, however, is not supported by the experience gained in these and previous experiments. It was seen in many instances that the Wolffian duct has a great growth energy and continues to grow and exist wherever even a small part of it can do so. This was particularly clear in the first series of experiments (1937), when in many instances the duct was damaged, intentionally or not, a short distance from its growing end. In all these cases the part caudal to the region of the operation grew normally and persisted, surrounded by a perfectly normal mesonephros. Moreover, the

scattered appearance of the independent nephrons under consideration speaks against the assumption of the temporary presence of a Wolffian duct. Hunt (1931), as well as Willier and Rawles (1931), described mesonephros differentiation in their explants of parts of the early chick blastoderm. These, however, do not prove self-differentiation because, as was pointed out by Boyden (1932) and Gruenwald (1937), they probably contained Wolffian ducts.

There is, at the present time, no possibility of definitely accounting for the differences in the behavior of the nephrogenic tissue in the present and previous series of experiments. As far as the author's own work is concerned, no difference in the technique can be made responsible, because self-differentiation also appeared in a few cases of the present series treated with electrolytic destruction in exactly the same manner as the embryos of the old series. Of external influences, only circumstances imponderable at the present time may be the cause of the discrepancy. There is, however, a possibility that differences between different breeds of chicks may be involved. To the author's knowledge no such differences have, up to the present time, been reported within one species.

Independent differentiation seems to occur occasionally in the dianephric blastema[1] of human embryos. In one case the author saw a branched epithelial tube near the inner inguinal ring, in the tissue adjacent to the caudal end of the mesonephros, in a 21-mm. human embryo (unpublished). A similar finding was reported by Meyer (1911); there can be little doubt that these were derived from the dianephric blastema, which is normally stimulated by neither the Wolffian duct nor the ureteric bud.

Induction of differentiation in the nephrogenic tissue by adjacent nervous tissue must be considered possible after the findings in three embryos of the present series. The results in these cases offer considerable difficulties to an analysis, since in all of them malformations probably not connected with the experiment accounted for the close proximity of nervous and nephrogenic tissues. Efforts are being made at the present time to work out a method of testing this possible influence of nervous tissue upon the nephrogenic blastema.

Nephrons differentiating far from the Wolffian duct but in close proximity to a spinal ganglion had been found in a maldeveloped 20-mm. human embryo (Gruenwald, 1941*a*). In this case the origin of nephrogenic tissue in its abnormal location at the lateral border of a persistent neural plate is problematic, but its differentiation could well be explained in accordance with the present findings by action of the adjacent ganglion.

A minor finding in the present series was that of external glomeruli on the ventral surface of the mesonephros in some of the experimental embryos. No interpretation can be attempted until controls have shown whether or not such glomeruli also occur in embryos with normal urinary organs.

According to the present state of our knowledge, important similarities, as well as certain differences, appear when we compare the mesonephric and metanephric blastemas. Both seem to be able to respond to inducing stimulation by the Wolffian duct by forming mesonephric nephrons. This indicates, as was mentioned above, that these two nephrogenic blastemas are potentially alike and that the inducing structure alone decides whether mesonephric or metanephric differentiation is to occur; the missing link

[1] The term "dianephric blastema" is herewith introduced to designate that part of the nephrogenic tissue between the mesonephric and metanephric blastemas which does not normally differentiate into nephrons (Schreiner's *Zwischenblastem*).

in this consideration, namely, induction of metanephros in the mesonephric blastema, can hardly be supplied experimentally. A similar comparison was made previously regarding the dianephric and metanephric blastemas of the human embryo (1939). It was shown in a case of agenesis of the ureteric buds in a 9-mm. human embryo that these blastemas were very much alike histologically when the metanephric tissue was not influenced by a ureteric bud. Since, as was pointed out previously (1938), the developmental mechanics of the urinary organs seems to be essentially alike in the chick and the human embryo, it might be suggested that the entire nephrogenic tissue (with the possible exception of the apparently more independent mother tissue of the pronephros) forms one essentially homogeneous mass as far as its potencies are concerned; its subdivision into mesonephric, metanephric, and dianephric portions is produced by contact with the Wolffian duct, the ureteric bud, or none of these, respectively. However, certain differences between those blastemas should not be overlooked. They appear particularly when these tissues remain uninfluenced by their normal inductors. In this event the mesonephric blastema cannot, if it does not undergo independent differentiation, be distinguished from the surrounding mesenchyme; the metanephric blastema, on the contrary, will form a denser mass of mesenchyme which can distinctly, although not sharply, be distinguished from the adjacent embryonic connective tissue. This difference is of minor importance compared with the fact that the metanephric blastema was never found to differentiate independently, whereas the mesonephric blastema did so to a considerable extent in many embryos of the present series. Even this, however, might well be a quantitative rather than a qualitative difference, concerning the degree of extrinsic stimulation necessary to elicit specific differentiation. These considerations suggest the assumption of one common nephrogenic potency in all parts of the nephrogenic tissue; more experience will, however, be necessary definitely to prove this conjecture.

SUMMARY

1. The Wolffian duct does not elicit differentiation of nephrons when in contact with the mesenchyme surrounding the axial organs or that of the body wall.

2. In contrast to previous results of the author's own experiments and those reported in the literature, self-differentiation of the mesonephric blastema in the absence of the Wolffian duct was found in the majority of embryos of the present series. The nephrons thus developed are more or less vestigial, and only part of the unstimulated tissue was found to have differentiated. Self-differentiation was never found in the metanephric blastema.

3. It is probable that nervous tissue can stimulate mesonephros differentiation in the nephrogenic tissue in the absence of the physiological inductor.

4. In one instance mesonephros formation was induced in the metanephric blastema by the displaced Wolffian duct. This suggests that there is only one nephrogenic potency which responds by mesonephros formation to stimulation by the Wolffian duct, and by metanephros differentiation to induction by the ureteric bud.

5. There is a slight difference in the behavior of the mesonephric and metanephric blastemas when they remain unstimulated by their physiologic inductors. The mesonephric blastema either forms independent nephrons or cannot be distinguished from the surrounding mesenchyme, whereas the metanephric blastema persists as a dense cell mass for a long time. It is then histologically very similar to those parts of the blastema in a normally developing kidney which are not yet differentiating into nephrons.

PLATE I

FIG. 7.—Embryo 709. The right Wolffian duct (×) is cut twice within the graft. No mesonephros differentiation was induced in the mesenchyme of the graft.

FIG. 8.—Embryo 752. The dilated right Wolffian duct (×) is shown within the graft, closely applied to a vesicle of nervous tissue. No mesonephros induction in the graft.

FIGS. 9, 10.—Embryo 725. The right Wolffian duct is shown within the graft (Fig. 9) with no mesenophros induction and, farther caudally in its normal surroundings (Fig. 10), with normal mesonephros formation.

FIGS. 11–13.—Embryo 741; see also Fig. 3. The Wolffian duct (arrow) is shown at the level of the graft accompanied by few mesonephric tubules (Fig. 11) or no mesonephros (Fig. 12), and caudal to the graft within a normal mesonephros (Fig. 13). The large cyst on the side of the operation is part of the graft.

PLATE II

FIGS. 14–19.—Embryo 724; see also Fig. 4. The right Wolffian duct is indicated by arrows or crosses. Its cranial portion is dilated (Fig. 14) and ends blindly (Fig. 15), after giving off a narrow duct (Figs. 15–17). This duct has not induced mesonephros formation in the graft (Figs. 16, 17). Its caudal part is curved medially and cranially again and has a dilated blind end. It has induced a mesonephros in the nephrogenic tissue of the host (Figs. 17–19), at least partly in its metanephric portion (Fig. 19). *U*, left ureteric bud surrounded by the metanephric blastema.

FIG. 20.—Embryo 727. The right Wolffian duct (arrows) has deviated from its normal course at the cranial border of the graft (not shown in the figure). It has not induced mesonephros formation on its course through the body wall.

PLATE III

FIGS. 21–23.—Embryo 733; see also Fig. 5. The right mesonephric blastema has partly differentiated in the absence of the Wolffian duct. It formed normal nephrons (Fig. 21, arrow), vestigial nephrons (Fig. 22), or inconspicuous solid cell cords (Fig. 23).

FIG. 24.—Embryo 773; see also Fig. 6. A large group of mesonephric tubules has formed in the absence of the right Wolffian duct caudal to the graft (left side of figure). Dorsal to the left mesonephros another group of tubules appears (arrow) in close proximity to a spinal ganglion and probably induced by it. The close proximity of nephrogenic and nervous tissues is due to a distortion of the embryo.

FIG. 25.—Embryo 775. (The growth of the right Wolffian duct had been experimentally obstructed at a higher level.) Owing to ourentery, the unstimulated right mesonephric blastema has come in contact with nervous tissue (*N*) and differentiated into tubules.

FIG. 26.—Embryo 766. An external glomerulus (arrow) is present on the ventral surface of the otherwise normal left mesonephros. On the other side the grafted tissues are seen; the right mesonephros is absent at this level.

FIG. 27.—Embryo 745. The right ureteric bud has failed to develop owing to experimental obstruction of the Wolffian duct. The unstimulated right metanephric blastema (*B*) may be compared with the normally developing left kidney (*K*). Silver impregnation of the reticulum fibers.

PLATE I

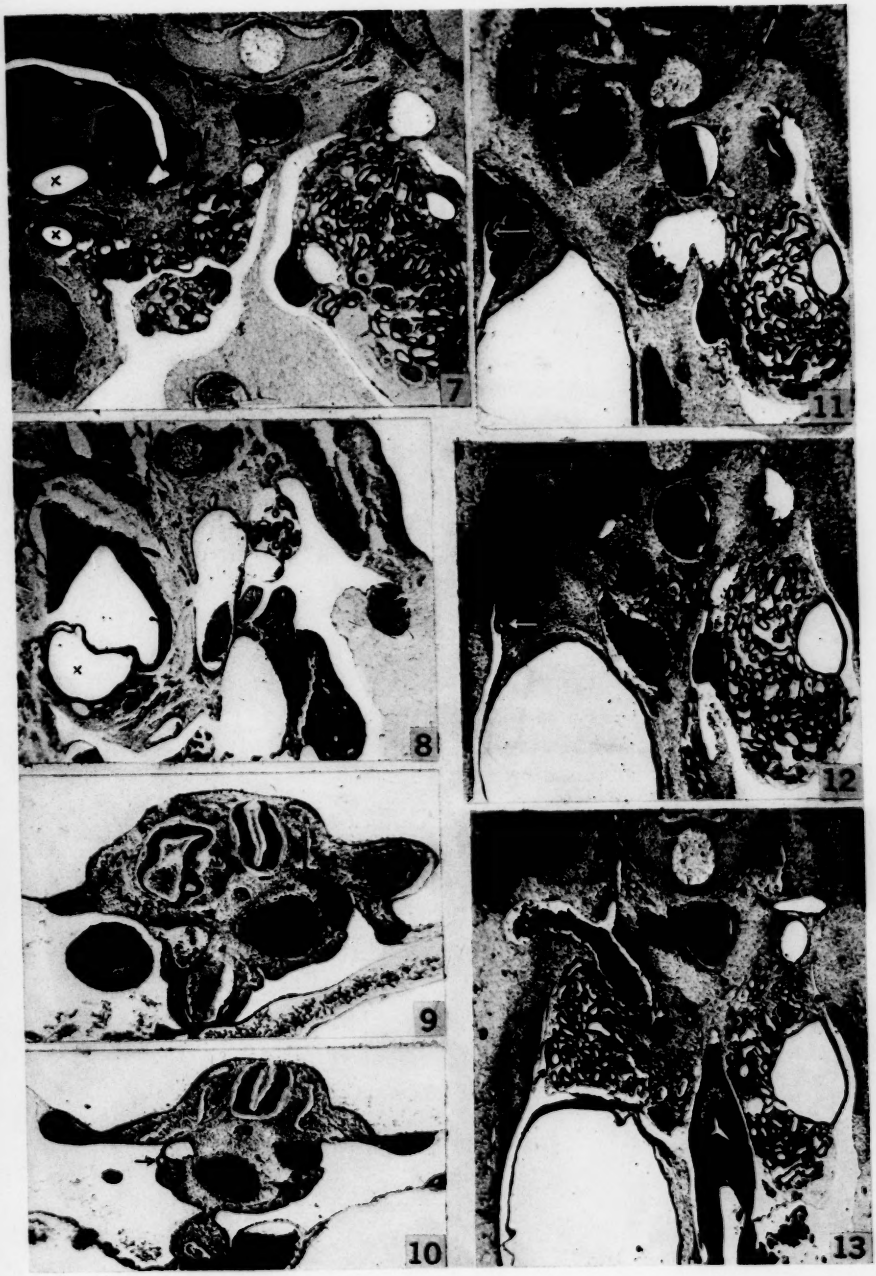


PLATE II

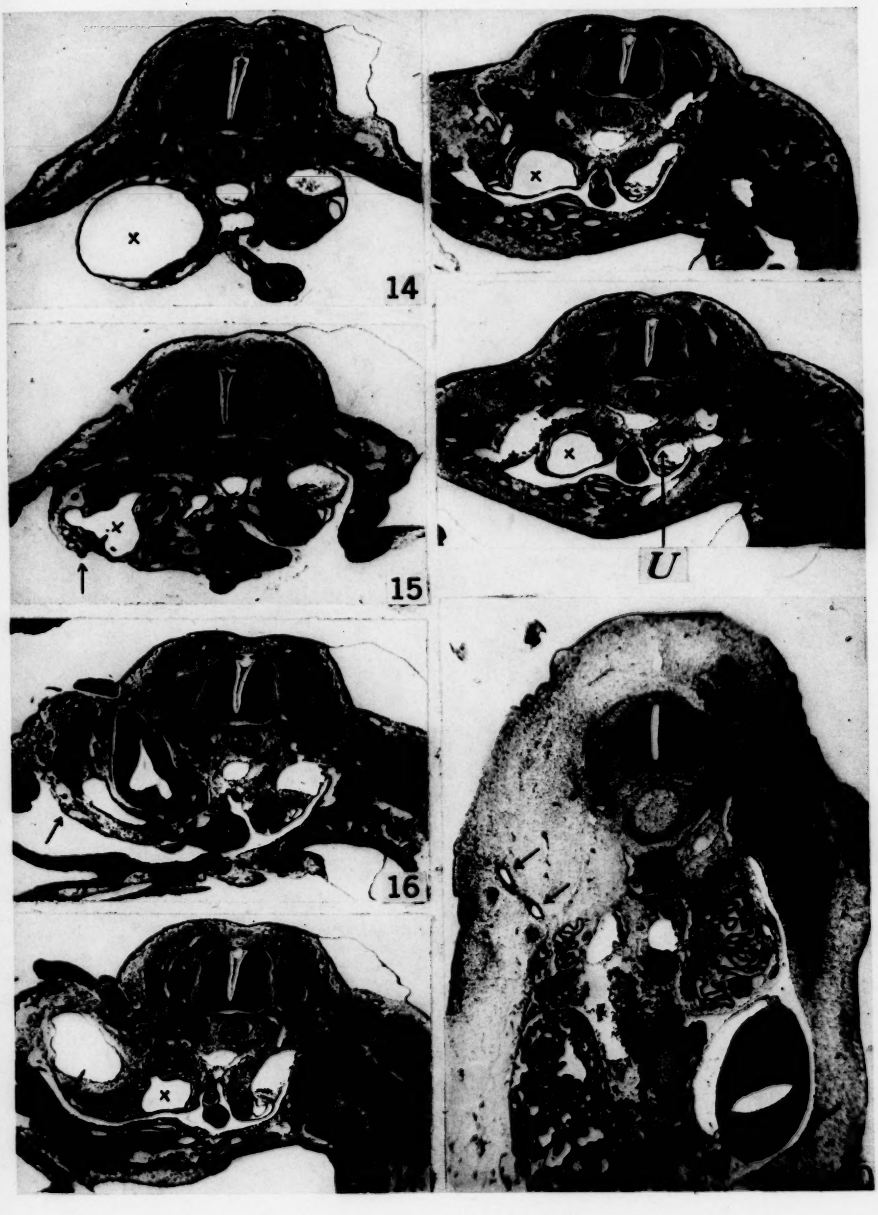
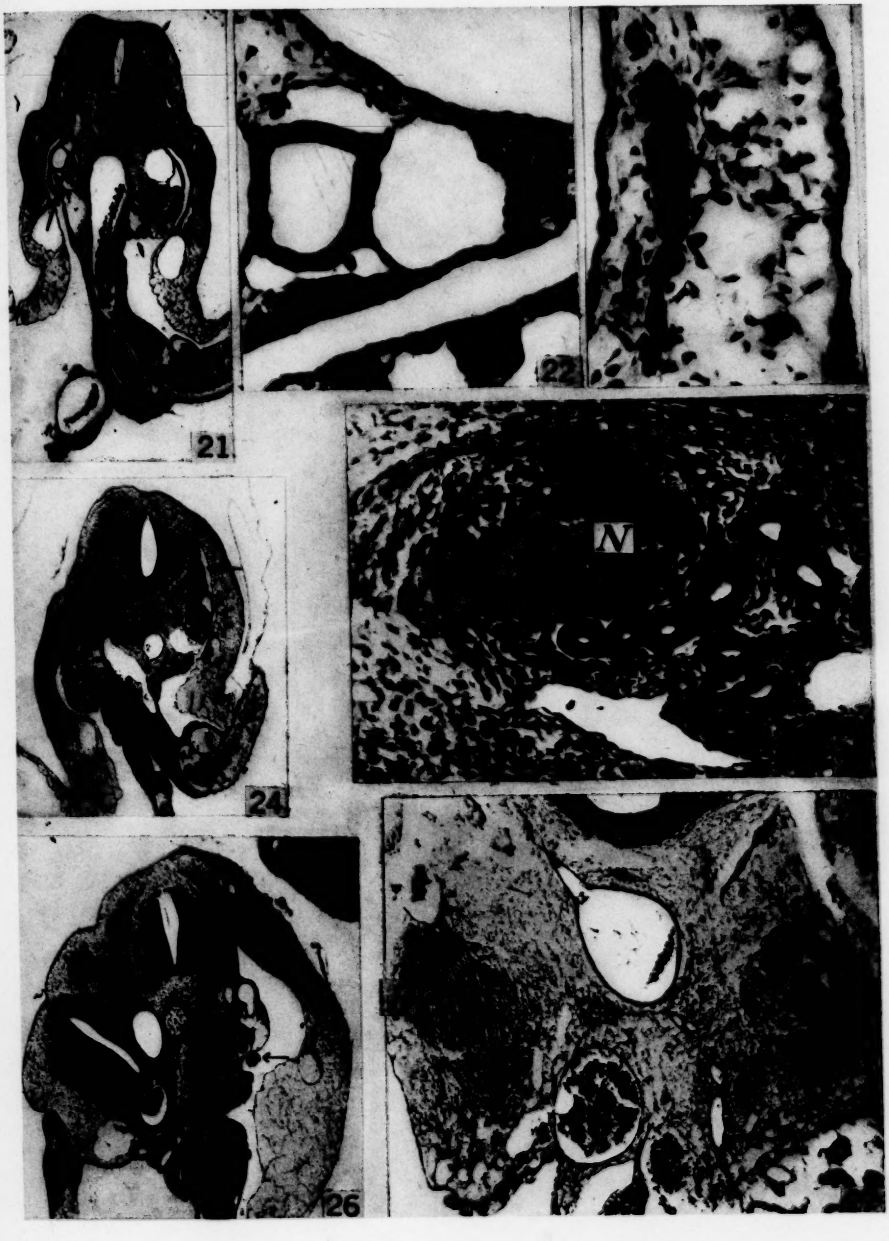


PLATE III



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NERVOUS CONTROL OF CHROMATOPHORES IN THE LEECH PLACOBDELLA PARASITICA

(Two plates)

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THE presence of chromatophores in certain annelids has been known for many years; yet very little work has been done on the physiology of color change in this phylum. Claparède, in 1870, described conspicuous yellow- and violet-pigmented cells in the Mediterranean polychaet *Nereis dumerilii*. His figures show the pigment of these cells both in the dispersed and in the concentrated state, but he did not observe changes in response to environmental factors. Later studies on this animal by Hempelmann (1911, 1939) have shown that the pigment cells can alter the color of the worm by their changes in shape. In darkness or in the absence of eyes the chromatophores are punctate, while illumination of the intact animal causes them to become stellate. Thus their function is probably as a shield against excessive illumination rather than as a response to the color of the background.

A similar situation is found in a number of leeches whose chromatophores have been studied. Johansson (1896)[1] showed that *Piscicola geometra* becomes dark colored in the light and pale in darkness. Stschegolew (1927) described the color changes in *Protoclepsis tessellata*, which can alter its tint from a translucent grayish green in darkness to a rather bright green with yellow spots when illuminated. Three types of chromatophores—brown, green, and yellow—are involved. Janzen (1932*a*) studied the distribution of, and mode of pigment movement in, the chromatophores of *P. geometra*. He verified Johansson's observations, and in a second paper (1932*b*) noted similar responses in the chromatophores of *Hemiclepsis marginata* and *Glossosiphonia complanata*. Wells (1932) reported that *G. complanata* has dark chromatophores whose pigment becomes dispersed in the light and concentrated in darkness but which are so scattered as to have little or no effect on the general coloration of the animal. Because there is no adaptation to background, Wells concluded that the chromatophores respond directly to the light, without control from within the animal.

The above workers all agree that annelid chromatophoral pigment becomes dispersed as a response to illumination and not to the color of the background. However, Kowalevsky (1900) has reported that *Haementeria* (= *Placobdella*) *costata* is commonly green when on water plants and brown when attached to turtles. Kowalevsky figures brown and green chromatophores, upon whose quantity and distribution the color of the leech depends, but he apparently did not observe pigment migration in these cells.

In the present paper there will be presented evidence that the chromatophores of at least one species of leech are under nervous control.

MATERIAL

Placobdella parasitica (Order Rhynchobdellida; Family Glossiphoniidae) has been used in the present study. This hardy leech, which was obtained in numbers from turtles

[1] Reference not available. Johansson's work is discussed by Stschegolew (1927).

(*Pseudemys*) shipped alive to Massachusetts from Louisiana, is well suited for observation because of its habit of lying appressed to the bottom or sides of the container. Animals from 2 to 4 cm. long are best, since they are thin enough to allow study with a dissecting microscope employing transmitted light and yet are large and thick enough to show good macroscopically visible color changes and to be operated upon.

The writer's attention was directed to the chromatophores by the marked color changes of leeches kept in the laboratory, where they were exposed to varying conditions of darkness and light. The pale appearance of animals after a period of darkness was in contrast to the dark appearance usually prevailing by day. The observations to be reported here were carried on from November, 1940, to August, 1941. About forty animals were studied.

Placobdella parasitica is of a brown or tawny color, with a mid-dorsal light stripe or row of spots, numerous light papillae, and, along the margin, white segmental and brown or gray intersegmental squares. The general coloration can pass from a condition in which the body, exclusive of the white areas, is a rather translucent light reddish brown to a state in which it is a more opaque dark brown with slate-gray marginal squares (Pl. I, Fig. 1). The color pattern is formed by three types of cells (Figs. 2–8):

1. In the light areas are found cells containing a pale yellowish, granular, reflecting substance (Figs. 2–4). Since these cells have not been observed to show changes in form, they will not be discussed further.

2. In the dark marginal squares and near the upper and lower body surfaces, as well as deep within the animal, are conspicuous chromatophores of variable size which in the punctate state appear black but which in the expanded state are seen to be green when observed with a dissecting microscope (Figs. 2–4). The green pigment is soluble in alcohol, as was also reported for that of *Haementeria* by Kowalevsky (1900).

3. Reddish-brown chromatophores occur over all of the brown or dark portions of the animal. In the expanded state they form a meshwork so fine that no cell centers are distinguishable, while even the processes were, for a time, overlooked as "ground color" (Pl. II, Figs. 6, 7). But in the punctate state the red chromatophores are conspicuous objects, although only about one-fourth the size of most of the green cells (Figs. 2, 5, 8). They occupy a more superficial position than do the green chromatophores, being close beneath the epidermis. Their pigment is not soluble in alcohol.[2]

REACTIONS OF THE GREEN CHROMATOPHORES

Most of the obvious color change is caused by the green cells, the reds acting to change the tint only slightly. Certain peculiarities in the behavior of the red chromatophores will be discussed in a later part of this paper. In the discussion of the gross color changes of *Placobdella*, reference is to the green chromatophores unless otherwise stated.

1. *The effects of darkness and light.*—When leeches are placed under illumination, they become dark in color, with the green chromatophoral pigment passing to the dispersed state, regardless of the color of the background. If the animals are placed in the dark, their green pigment becomes concentrated, causing them to assume the light-colored phase. This dispersion of pigment in light and concentration in darkness is in agreement with the findings of all previous workers who have examined the changes in annelid chro-

[2] Satisfactory mounts, showing both types of chromatophores, may be obtained by fixation in 9 parts of saturated corrosive sublimate plus 1 part of formalin, washing, and clearing in dioxane. Since the muscles of cut pieces contract strongly, flattening of the tissue between glass slides soon after the start of fixation is often helpful.

matophores. The time required for the green pigment to pass from the dispersed to the concentrated state, or vice versa, is about an hour for normal, resting animals at 18°–20°C.

2. *The effect of background.*—The responses to light mentioned above are the same whether the animals are on white or on black backgrounds.

3. *The effect of decapitation.*—Leeches blinded by removal of the head with its two eyespots are still able to change color. However, a headless leech changes color much more slowly than a normal animal, often taking more than a day to make the full shift. This applies to animals that have had several days in which to "recover" from the operation. Since it is well known that many annelids are light-sensitive over most of the body, it is possible that the slowness of the color change in the headless leech is the result of the loss of the eyes, leaving only the scattered photoreceptor elements of the body.

If a leech that has been kept in darkness until it is fully pale in color is quickly decapitated and returned to darkness, it rapidly becomes very dark in color, just as if strongly illuminated. This condition lasts for 24 hours or more, after which the leech slowly returns to the pale, unilluminated state. Such a reaction must indicate that the green chromatophores are responding to something other than the direct action of light. If, after the headless leech has become pale (in darkness), a further piece is cut from the anterior end, there occurs a similar darkening of almost as long duration as that following decapitation. Here it cannot be a result of the sudden removal of eyes or anterior ganglia but would rather seem to be the result of the injury to the nerve cord, which might cause dispersion of the green pigment by means of nervous impulses to the chromatophores themselves or to some source of chromatophorotropic hormone.

4. *Differential color changes.*—Attempts were made to produce differences in the state of the green chromatophores in different parts of the same animal. Two methods were successful, both of them indicating that these chromatophores are not under hormonal control.

a) Effects of partial shielding: Leeches were kept in darkness until fully pale in color. They were then illuminated, with portions of the body shielded by strips of opaque paper. With the rear half of the body covered, the pigment of the green chromatophores of the front half passed to the dispersed state as rapidly as usual, while that of the rear showed little or no dispersion. With the anterior half covered, the reverse effect could be obtained, but on a less marked scale. Apparently the anterior half of a leech is more sensitive to light than is the posterior half—a fact doubtless related to the presence of eyes on the head. This type of experiment indicates that a chromatophorotropic hormone is not involved, but it does not tell whether the green chromatophores are independent effectors or are under the control of nerves. Since leeches are probably more or less light-sensitive over the entire body, illumination of the photoreceptors on the body might possibly affect the chromatophores in the same general region by way of segmental reflexes.

b) Effects of illumination following nerve-cord transection: The immediate effect of transection of the nerve cord (animal immobilized on ice) is a general darkening, such as follows decapitation. After recovery the responses to light continue normally in the majority of animals, indicating that the control of chromatophores, if nervous, may be exerted by segmental reflex mechanisms. Certain animals, however, show greatly lessened color changes posterior to the level of nerve-cord transection. Figure 9 shows one such animal which, on being brought from darkness into the light, failed to darken (become light-adapted) in the posterior part of the body. The presence of the eyes would

seem adequate to account for the greater responsiveness of the anterior region, while the failure of response posterior to the break in the nerve cord indicates that the central nervous system may be concerned with the passage of impulses from cephalic photoreceptors to the chromatophores of the body. Furthermore, the failure of the green pigment to become dispersed in a part of the body when the whole animal is exposed to light is positive evidence that, in some cases at least, pigment dispersion in the green chromatophores is not caused by the direct action of light. Such cases may possibly result from a low level of photosensitivity or of segmental reflex activity in the posterior body regions.

c) Effects of electrical stimulation after nerve-cord transection: Faradic stimulation of the intact, dark-adapted (pale) leech produces extreme darkening (the light-adapted state), which may persist as long as a day, even in darkness. Leeches whose nerve cords had been cut were kept in darkness until fully pale in color. They were then stimulated faradically at the head or the tail end and immediately returned to darkness. After half or three-quarters of an hour they were found to be darkened in the end stimulated, but on the other side of the cut in the nerve cord they remained pale. Microscopic examination showed that the pigment of the green chromatophores was dispersed in the stimulated end, as far as the cut, but was concentrated in regions beyond that point. This response to electrical stimulation is obtained in almost all trials, whereas well-marked differential responses to light by animals with cut nerve cords are not commonly seen. Since light is apparently more effective than localized faradic stimulation in producing darkening on both sides of a break in the nerve cord, it seems likely that the difference is due to scattered photoreceptors in the rear part of the body. The fact that response to electrical stimulation consistently stops at the level of nerve-cord transection indicates that impulses to the green chromatophores are being carried in the central nervous system. The appearance of a leech darkened differentially by faradization of the head is similar to that shown by the animal in Figure 9, which had responded to light.

It may be argued that the blocking of color change by nerve-cord transection is not entirely satisfactory evidence for the nervous control of chromatophores, since important blood vessels are cut in the operation. This is inevitable because the nerve cord actually lies within a blood sinus. It might then be claimed that the effects described are due to the restriction of the spread of a chromatophorotropic hormone in the blood stream. While such a possibility should not be forgotten, it appears unlikely because (*a*) intact animals show differential responses of the greens as the result of partial shielding from light and (*b*) the regions posterior to the level of nerve-cord transection, while completely unco-ordinated with the muscular reactions of the anterior parts, show no sign of any injurious effects that might be expected to result from stoppage of their blood supply.

5. *Effects of "disturbing factors."*—In the course of these observations there have been noted situations in which animals do not react in the ways described above. For instance, animals in the dark are sometimes found to have their green chromatophores partly expanded instead of in the usual punctate state. Such animals generally show high activity and do not rest quietly, as do most leeches under favorable conditions. Various minor factors may produce this activity. A recent change of water (often involving a shift in temperature), attempts to apply drugs or to make injections, lack of oxygen, or excessive summer temperatures of about 30° C. or more have all been observed to make animals so "restless" that the punctate condition of the green chromatophores may not be obtained even after a long period in darkness. For this reason, experiments with drugs and anesthetics have been inconclusive. For instance, chloretone anesthesia causes dispersion

of the green pigment. Yet it cannot be stated that the dispersion is caused by the blocking of nervous activity, since darkening starts while the leech is in a highly irritated state, before the anesthetic effect is attained.

6. *Effects of isolation of chromatophores.*—Attempts to isolate chromatophores from all influence of nerves by the removal of small pieces of tissue have given no conclusive results. In such isolated tissue (studied in a solution previously found suitable for a physiological preparation of leech muscle) the chromatophores remain in a more or less dispersed state indefinitely, even in darkness, possibly because of some stimulus resulting from cutting. Partly because of the difficulty of separating skin from underlying muscle in *Placobdella*, attempts to transplant bits of skin and subdermal tissue have so far been unsuccessful.

REACTIONS OF THE RED CHROMATOPHORES

While the reactions of the green chromatophores are, on the whole, consistent, those of the red chromatophores are still rather obscure. Certain generalizations may be made:

1. *Independence of responses of red and of green chromatophores.*—In the majority of resting animals observed the red pigment is dispersed, regardless of the background, illumination, or the state of the green chromatophores. In many cases, however, the red chromatophores have been observed to be punctate, again without regard to the condition of the green cells. Examples of the independence of the two types of chromatophores are shown in Figures 2, 3, 4, 7, and 8.

2. *Effects of "disturbing factors."*—When the red pigment is in a dispersed condition, the application of such stimuli as changes of water, rough handling, drugs, electric shocks, or sudden exposure to light frequently causes it to pass to the concentrated state for a short time, usually less than an hour, after which it becomes dispersed again if the animal is quiet. At high summer temperatures (30° C. or more), which tend to make the leeches quite active, the red pigment may be concentrated over long periods.

3. *Effects of electrical stimulation after nerve-cord transection.*—When electrical stimuli are applied to animals which have had their nerve-cords cut and in which the red chromatophores are expanded, they usually become punctate, but only in that half of the animal which has been stimulated. The occurrence of this effect is somewhat unpredictable; but the fact that, when it does occur, it is limited by the level of nerve-cord transection, makes it appear likely that the red chromatophores of *P. parasitica* are under nervous control. Electrical stimulation of animals having punctate red cells does not cause the red pigment to become dispersed. Thus the red pigment is probably concentrated by nervous action, in contrast to the green, which is dispersed by nerves. The absence of a consistent relationship between the responses of the two types of color cells makes it possible that they are supplied by different nerves, but this point requires further study.

OBSERVATIONS ON OTHER SPECIES

Four other species in the family *Glossiphoniidae* have been studied briefly. The responses of *G. complanata*, as reported by Wells, have been verified. No pigmentary responses have been detected in *Glossiphonia stagnalis*. A leech believed to be *Placobdella picta* shows reddish and green chromatophores quite comparable in response and form to those of *P. parasitica*. Two specimens of *Placobdella rugosa* have been found to possess only reddish-brown chromatophores, similar in appearance to those of that color in *P. parasitica*. The reddish pigment of *P. rugosa* is dispersed in light and concentrated in

darkness. Apparently the red chromatophores of this leech react much as do the green ones of *P. parasitica*. It does not, at present, seem wise to try to homologize the red chromatophores of these two species, since in *P. rugosa* they carry on the entire color change, while in *P. parasitica* they are of secondary importance.

DISCUSSION

The results given above indicate that the green chromatophores of *P. parasitica* are capable of being dispersed by nervous activity, whether this state of activity be set up by light or by various other factors of a stimulating nature. It is likely that the stimulus of light results in an increased flow of impulses from the photoreceptors, via the central nervous system, to the green chromatophores, causing the dispersal of their pigment. The cause of the punctate condition of the green cells is not wholly clear. On the basis of present evidence it appears probable that the green chromatophores are punctate in the unstimulated or "resting" state.

The tendency of the red chromatophores to become punctate following stimuli of various sorts may mean that their "resting" state is in the expanded condition. If this is so, the red chromatophores show considerable ability to adapt, since they are commonly found expanded in animals that have been quiescent for some time, regardless of the degree of illumination and of the condition of the green chromatophores.

The results reported in this paper cast doubt on the conclusions of Wells (1932), who has attempted to fit the responses of *G. complanata* into the type of "primary"[3] or "direct" responses because of the fact that the chromatophores were observed to expand in light and to contract in darkness regardless of the color of the background. Since this leech does not adapt to background, Wells has assumed that it "apparently presents the primary mode of response alone and uncomplicated by the secondary." However, the mere fact that the chromatophores of *G. complanata* react to illumination rather than to background is not, in itself, sufficient evidence for Wells's claim that the response is a "primary" one. Although, in many animals, "secondary" pigmentary responses involve adaptation to background, the response of the leech to illumination is probably as "adaptive" as are responses to background. Janzen (1932*b*) has presented evidence that the dispersion of pigment in leeches serves as a protection against the injurious effects of excessive illumination. In *P. parasitica* the response to illumination is largely, if not wholly, a "secondary" one, since it is mediated by the central nervous system. It would be of interest to know whether or not the nervous control of the response to light has been superimposed upon a "primary," but now obscured, direct response of the chromatophores to light.

SUMMARY

1. The leech *P. parasitica* possesses green and reddish-brown chromatophores, the pigments of which, by their dispersion and concentration, can alter the coloration of the animal.

2. There is no adaptation to the color of the background, but *Placobdella* darkens under illumination and becomes pale in darkness.

[3] As generally understood, a "primary" response of a chromatophore is an expansion caused by the direct action of light, without any influence mediated by visual organs or nervous system. On the other hand, a "secondary" response is considered one mediated through the eyes and the nervous or endocrine system.

EXPLANATION OF FIGURES

The following key applies to Plates I and II.

Y: Area of cells containing light-yellow reflecting substance
GC: Area containing mostly concentrated green pigment
GD: Area containing mostly dispersed green pigment
RC: Area containing mostly concentrated red pigment
RD: Area containing mostly dispersed red pigment

PLATE I

FIG. 1.—*A*, a living, dark-adapted leech, photographed by a combination of transmitted and reflected light. *B*, the same animal after an hour in the light, photographed with the same exposure used in *A*. Note the greater translucency in the dark-adapted state. The negatives of *A* and *B* were printed as one on the same piece of paper. ×2.

FIG. 2.—Fixed preparation of a piece of the margin of a dark-adapted leech, with both green and red chromatophores punctate. Approx. ×50.

FIG. 3.—A piece of a leech, removed and fixed 35 minutes after exposure to light. Green chromatophores half-expanded; red chromatophores punctate. Approx. ×50.

FIG. 4. A piece of the same animal as in Fig. 3, removed and fixed 70 minutes after exposure to light. Green pigment maximally dispersed; red concentrated. (The expansion of the green chromatophores in this animal was intensified by the stimulus of removal of pieces at intervals after exposure to light.) Approx. ×50.

PLATE II

FIG. 5.—Red chromatophores fully punctate (fixed preparation). The green chromatophores have been dissolved out in alcohol. Approx. ×50.

FIG. 6.—Red chromatophores fully expanded. Green chromatophores dissolved as in Fig. 5. Approx. ×50.

FIG. 7.—Fixed preparation of a piece of a leech dark-adapted at 3° C. Green pigment concentrated; red fully dispersed. Approx. ×50.

FIG. 8.—Piece of the same leech as in Fig. 7, fixed after dark-adaptation at about 28° C. Green pigment concentrated; red mostly concentrated except for a narrow zone of dispersion. The explanation of this unusual condition is not known. Approx. ×50.

FIG. 9.—A leech (head at left) with transected nerve cord, which had been allowed to become uniformly pale in darkness and then had been illuminated for 1¾ hours. Note the failure to darken (become light-adapted) posterior to the level of nerve-cord transection. ×2.

PLATE I

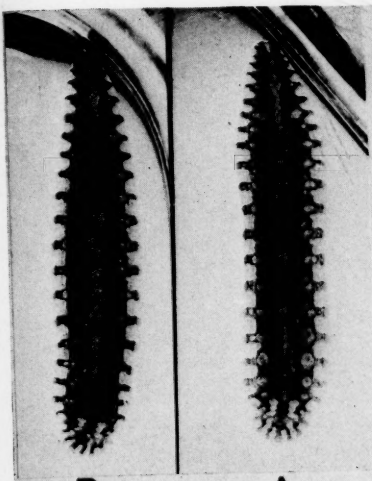

FIG. 1

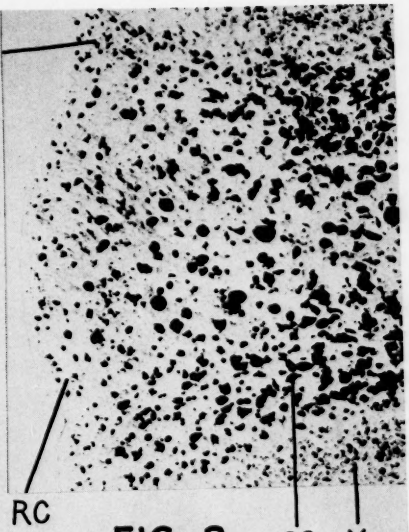

FIG. 2

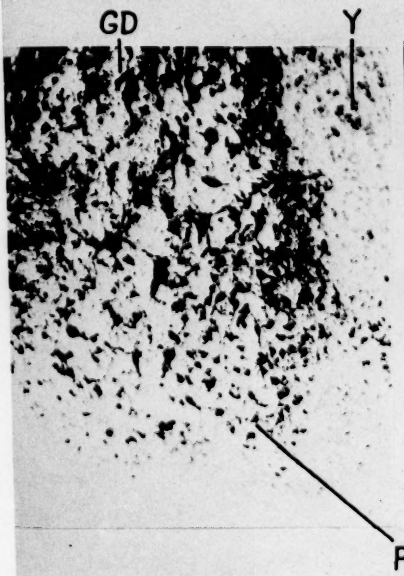

FIG. 3

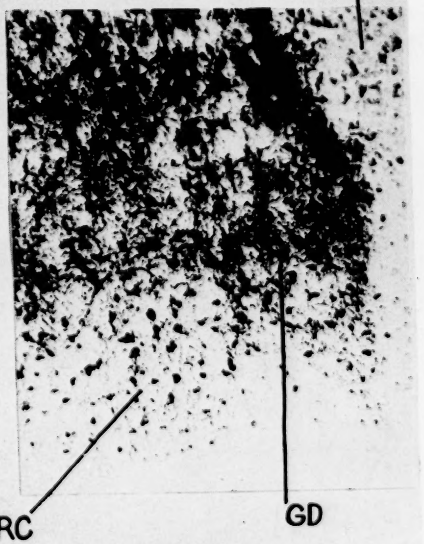

FIG. 4

PLATE II

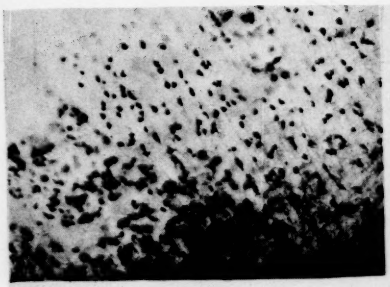

FIG. 5

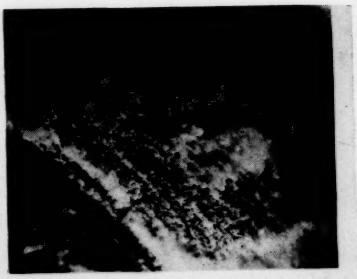

FIG. 6

FIG. 7

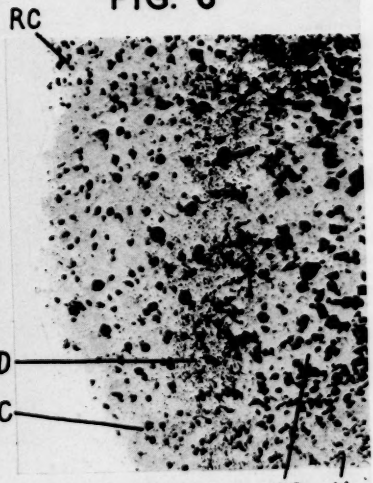

FIG. 8

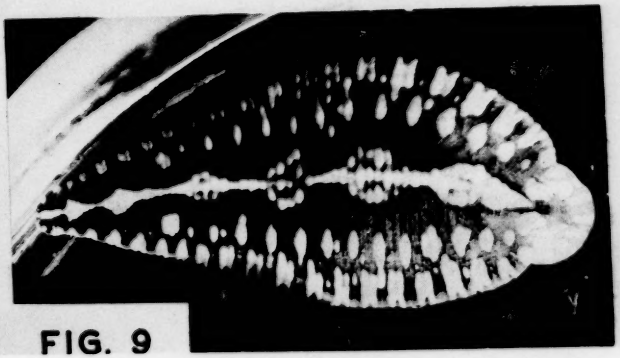

FIG. 9

3. These gross color changes are due mainly to the green chromatophores, which are punctate when the animal is resting in darkness but which become expanded when the leech is exposed to light or to various other factors of a stimulating nature.

4. The dispersion of the green pigment is brought about by nervous action mediated through the central nervous system.

5. There is no evidence of a direct effect of light on the green chromatophores.

6. The red chromatophores react independently of the green ones.

7. Although the responses of the red chromatophores are rather irregular, it seems probable that their pigment is concentrated by a central nervous mechanism.

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EFFECTS OF CERTAIN DRUGS AND IONS ON THE OYSTER HEART[1]

(Fifteen figures)

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EXTENSIVE experiments on the effects of ions on the oyster heart have been performed by Walzl (1937, 1938). He found that excess of calcium in the perfusion fluid decreased the frequency and increased the amplitude of contraction and that solutions containing only calcium chloride caused diastolic arrest. An excess of potassium caused a slight increase in frequency and a marked decrease in extent of relaxation. In solutions containing a great excess of potassium or in solutions containing only potassium chloride the heart was arrested in systole. These effects are apparently opposite to the well-known action of potassium and calcium on the vertebrate heart, and Walzl concluded that in the oyster heart calcium affects primarily excitation and that potassium acts primarily on the contractile mechanism.

The effects described by Walzl (1937) are those that appear immediately following the introduction of the different perfusion solutions involved. It is possible that these effects are caused by the combined action of the newly introduced perfusion fluid and traces of the previous perfusion fluid still remaining in the heart. The purpose of the present paper is to describe experiments in which the effects of perfusing with various solutions were observed over an extended length of time.

MATERIALS AND METHODS

All experiments were performed on the isolated and perfused ventricle of *Ostrea virginica* (*Gryphaea virginica*, according to Nelson, 1938). Narragansett Bay oysters, obtained in a fresh condition, served as a source for this material. The method of excising and perfusing the ventricle was similar to that described by Walzl (1937). Perfusion was carried out at 25° ± 1° C., and the hydrostatic pressure of the perfusion fluid was kept at 6 cm. of sea water.

Recording of the heart beats was accomplished photographically. The heart was connected to a torsion lever, the construction of which is indicated in Figure 1. A light source and optical system was so arranged that the arm of the lever cast a shadow which was focused on the slit of a recording camera containing 35-mm. Bromide paper (Eastman Kodak, Grade R). The use of a torsion lever and of photographic recording has certain advantages over the smoked-drum kymograph method; friction is eliminated, and the force against which the heart must work can be adjusted easily by changing the

[1] This paper is based on part of a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Brown University. The author wishes to express his gratitude to Professor Ivon R. Taylor for much valuable advice.

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torque on the torsion wire. In the present work the tension was kept constant throughout each experiment, although the value of the forces exerted on different hearts varied from 0.5 to 1.0 gm. The contractions were not quite isotonic, since the torque on the wire increased as the lever was displaced by the heart. In moving the lever an amount which resulted in a 35-mm. displacement of the recorded shadow, the heart increased the force against which it contracted by about 0.1 gm.

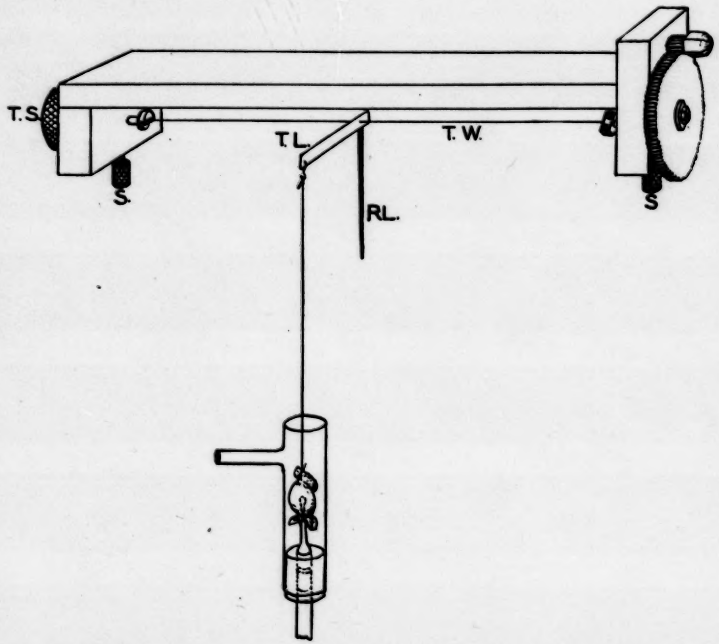

FIG. 1.—Diagram showing the torsion lever and perfusion chamber with the heart in position. The frame of the torsion lever is constructed of steel, ⅜ inch in thickness, 1 inch wide, and with an over-all length of 6 inches. The torsion wire (*T.W.*) is of constantan and is 0.0059 inch in diameter. The torsion lever (*T.L.*) is a strip of aluminum 1¼ inches in length; it is fastened to the torsion wire with de Kotinsky cement. The recording lever (*R.L.*) is a 2-inch length of No. 22 copper wire coated with black paint. The tension on the wire may be adjusted by the thumbscrew (*T.S.*), and the torque is regulated by the knob at the right. The screws (*S*) are setscrews.

As a normal perfusion fluid, sea water adjusted to the desired pH or van't Hoff solution was used. The concentrations of the salts used in the van't Hoff solution were as follows:

Sodium chloride	0.454 M	Magnesium chloride	0.035 M
Potassium chloride	0.010	Magnesium sulphate	0.017
Calcium chloride	0.010	Sodium bicarbonate	0.0001–0.001

In certain experiments solutions containing only one salt were used. These were made isosmotic with van't Hoff solution by determining the freezing-point of the latter

and then calculating for each salt the concentration necessary to produce an equal freezing-point. The salts used were of "Baker's Analysed" or Baker and Adamson "Reagent" quality. Deliquescent chlorides were standardized by the Volhard method. Water distilled from a tin-lined still and redistilled from a glass still in the presence of potassium permanganate was used in making up all solutions.

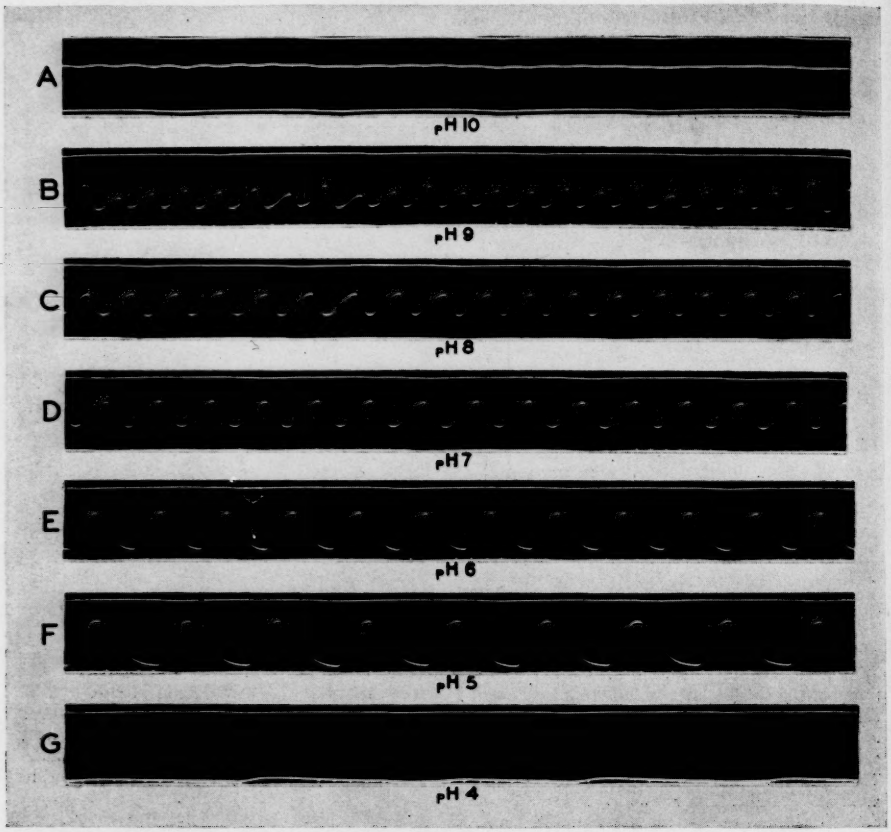

FIG. 2.—The effect of varying the pH of the perfusion medium. Each record was taken after 30 minutes of perfusion with sea water having the pH value indicated. In this and in all subsequent records an upward deflection indicates systole.

In some experiments drugs which altered the pH of the perfusion fluid were used. In such cases care was taken to readjust the pH to that of the control medium. The necessity for doing this is shown by the results of fourteen experiments in which the pH activity of sea water was varied over the range in which the heart would beat. The pH of the sea water was adjusted by the addition of sodium hydroxide or hydrochloric acid and was measured colorimetrically by means of LaMotte indicators

and color standards. Corrections for salt error were estimated from the factors given by Harvey (1928); so the pH values reported are probably accurate to within 0.15 pH unit. The concentrations of the acid and base used to adjust the pH were such as to produce no significant osmotic effects.

Records from a typical experiment on the effects of pH are shown in Figure 2, and in Figure 3 the rate of beat is shown as a function of pH. Below pH 4 diastolic arrest occurs. As the pH is increased, the rate of beat increases in a regular manner up to pH 9. Above this value heartbeats tend to become irregular, and they stop in a contracted state at a pH of about 10. A tendency toward irregularity at pH 9 is evident in the record shown in Figure 2. The amplitude of contraction is at a maximum at pH 5–6.

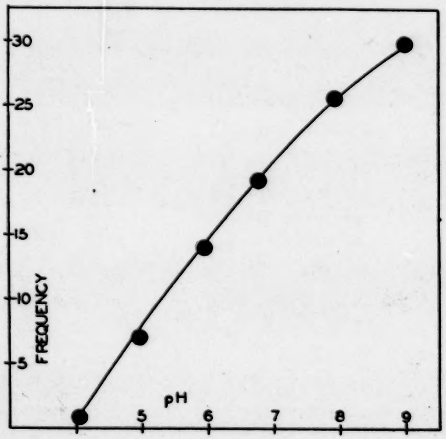

FIG. 3.—The effect of the pH of the medium on the frequency of heart beat. The results shown are based on average values of fourteen experiments. The frequency is given in beats per minute.

How much of the "pH effect" described above is caused by changes in carbon dioxide tension in the solution or by changes in the ionization of calcium salts was not determined. It is obvious, however, that pH control is important in experiments of the type reported in the following pages.

In the experiments described below, the following preliminary procedure was carried out. After the dissected preparation was set up in the perfusion chamber and the torsion lever adjusted so as to exert the desired tension, the heart was perfused for half an hour in the normal perfusion fluid (sea water or van't Hoff's solution) before any experimental substances were added. This precaution was taken to allow the heart the opportunity to become adjusted to an artificial medium. Occasionally hearts failed to beat, or they developed irregular beats. Such hearts were discarded, and only hearts which showed a strong regular beat during the preliminary perfusion were used for experimentation.

RESULTS

For each type of experiment several different hearts were used. The records shown and the results described are typical, although individual differences in threshold and in quantitative response to drugs appeared. The pH of all perfusion fluids was 7.3 ± 0.2.

a) *The effect of potassium chloride.*—When van't Hoff solution is substituted by an isosmotic solution of potassium chloride, the heart is immediately arrested in systole (see Fig. 4). The extent of contracture is greater than that of normal systole. This extreme contracture is less than a minute in duration and is followed by a gradual relaxation. After 10 minutes of perfusion with isosmotic potassium chloride the heart has relaxed to the normal diastolic level, and at the end of 30 minutes it is still further re-

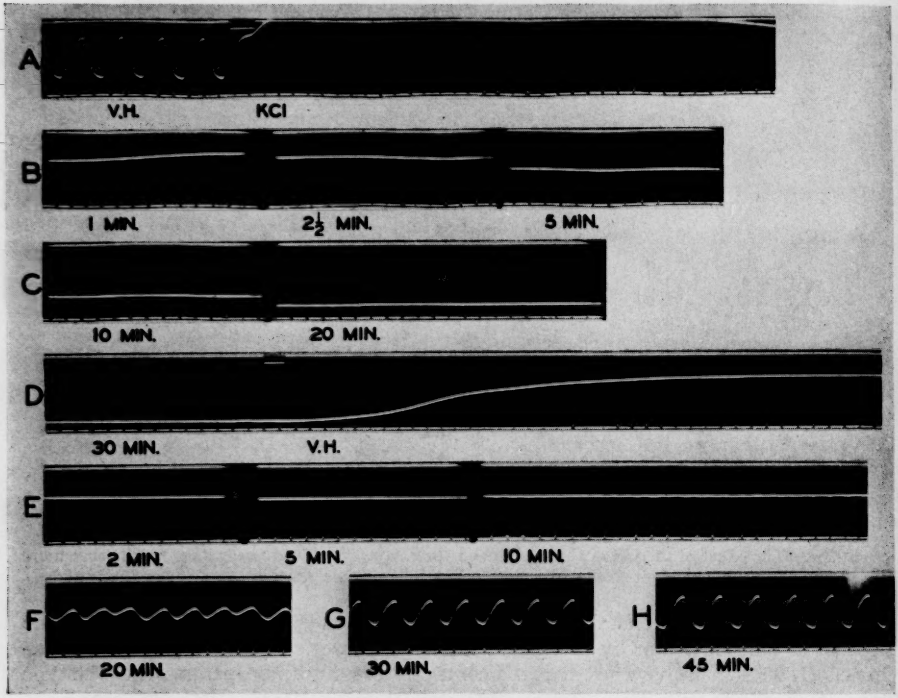

FIG. 4.—The effect of potassium chloride on the heart. *A*, the heart is beating normally in van't Hoff solution; at the signal potassium chloride, isosmotic with van't Hoff solution, was introduced. *B*, *C*, showing the gradual relaxation of the heart in potassium chloride. *D*, showing the extreme relaxation after 30 minutes in potassium chloride; at the signal van't Hoff solution was introduced. *E*, *F*, *G*, *H*, showing the recovery of the heart in van't Hoff solution.

laxed. The heart at this time has an appearance of extreme dilatation. Upon being returned to van't Hoff solution the heart immediately responds with a contracture or marked increase in tonus. Several minutes are required for the appearance of rhythmic action, but eventually the normal beat is restored.

b) *The effect of sodium chloride.*—The effect of isosmotic sodium chloride is similar, in general (as shown in Fig. 5), to that of potassium chloride. However, the initial contracture produced by sodium chloride is neither so rapid in appearing nor so great in

magnitude as that produced by potassium chloride. Rhythmic beating of greatly reduced amplitude persists for nearly a minute, but the extent of relaxation decreases progressively until the heart comes to rest in a contracture at the normal systolic level. Following this, a gradual relaxation occurs; at the end of half an hour the heart is relaxed more than in normal diastole and is greatly dilated. Complete recovery occurs in about an hour when the heart is reperfused with van't Hoff solution.

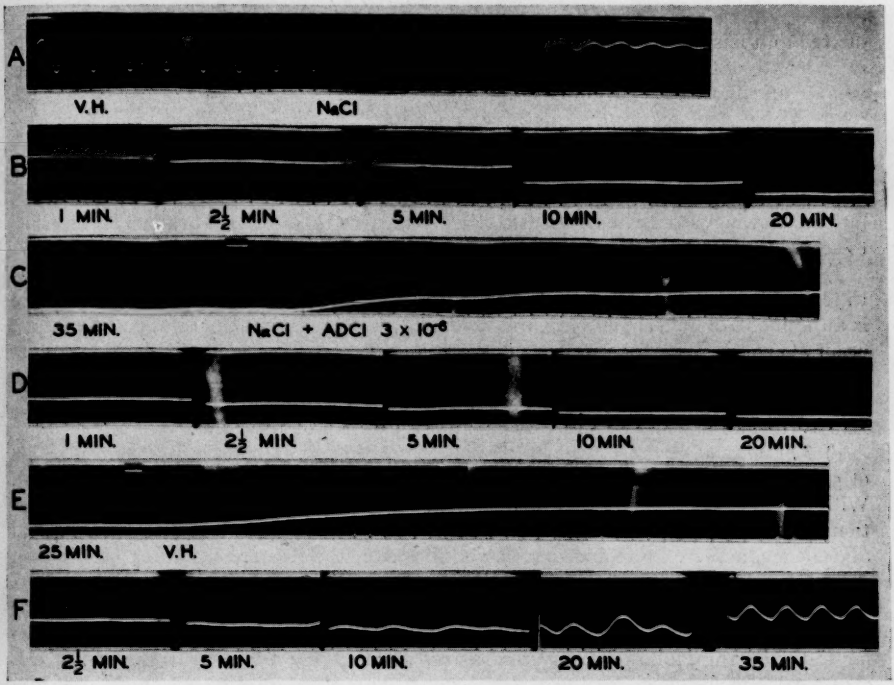

FIG. 5.—The effect of sodium chloride on the heart, and the effect of adrenalin in isosmotic sodium chloride. *A*, at the signal sodium chloride, isosmotic with van't Hoff solution, was introduced. *B*, showing the gradual relaxation in sodium chloride. *C*, at the end of 35 minutes the heart is relaxed so greatly that the shadow of the lever is coincident with the lower margin of the record; at the signal isosmotic sodium chloride containing adrenalin chloride $3:10^6$ was introduced. *D*, showing the lack of persistence of the adrenalin effect. *E*, at the signal van't Hoff solution was introduced. *F*, showing recovery of the heart in van't Hoff solution.

c) *The effect of calcium chloride.*—When the heart is changed from van't Hoff solution to an isosmotic solution of calcium chloride, the immediate effect is arrest in the relaxed state, usually at the normal diastolic level. This is followed by a few random beats and the development of a contracture which reaches its maximum height within a minute or two and which seldom attains a level higher than that of normal systole. The heart gradually relaxes, and by the end of half an hour it has attained the normal diastolic

level, but it seldom relaxes further. These effects are shown in Figures 12, 13, 14, and 15. The extreme dilatation characteristic of the relaxation in potassium chloride and in sodium chloride is absent. When returned to van't Hoff solution, the heart recovers its normal beat.

d) *The effect of van't Hoff solution lacking calcium chloride.*—Changing the heart from van't Hoff solution to van't Hoff solution lacking calcium chloride results in an immediate increase in diastolic tone; within a few seconds, arrest in a semicontracted state occurs. A gradual relaxation follows, until at the end of 30 minutes the heart is extremely dilated and has the appearance possessed by hearts which have been perfused for the same length of time in potassium chloride or sodium chloride. The effect of calcium-free van't Hoff solution is shown in Figures 9, 10, and 11.

e) *The effect of sea water containing added calcium chloride.*—When sea water containing 11 or 12 gm. of added calcium chloride per liter is substituted for normal sea water, the heart shows a distinct decrease in rate of beat and usually a slight increase in the amplitude of contraction (Fig. 7). The amplitude returns to normal in a few minutes, but the slowing is persistent. Hearts quickly recover their normal beat when returned to sea water.

f) *The effect of excess calcium on adrenalin action.*—Adrenalin chloride $1:10^6$ in sea water produces an effect similar to that caused by addition of excess potassium. There is an immediate increase in tone and rate; and, within a minute or two, systolic arrest occurs (Fig. 6). When sea water containing 12 gm. of added calcium chloride per liter and adrenalin chloride $1:10^6$ is introduced to a heart beating normally in sea water, the tone is increased and the rate is slowed, but the heart continues to beat rhythmically. Excess calcium appears to partly antagonize the action of adrenalin.

g) *The effect of excess calcium on the action of digitalin.*—Digitalin in a concentration of $5:10^5$ produces an irregularity of rhythm and tendency toward contracture (Fig. 7). With excess calcium chloride in the medium, however, digitalin in the same concentration does not exert these effects. Although the contractions are slower than normal and although there is an increase in systolic tone, the heart continues to beat with perfect rhythm.

h) *The effect of excess calcium on quinine action.*—The result of introducing quinine sulphate 1:1,000 in sea water to a heart beating normally in sea water is a marked slowing of the rhythm, followed by systolic arrest within 2 or 3 minutes. In the presence of excess calcium, however, the heart continues to beat rhythmically but shows great increase in both diastolic and systolic tone. This increase of tone is so great, in fact, as to cause the shadow of the recording lever to be almost completely displaced from the recording paper; for this reason no record of this type of experiment is included in the figures.

i) *The effect of excess calcium on nicotine action.*—In a concentration of $1:10^4$ nicotine reduces the amplitude of contraction without seriously affecting the rhythm; there is practically no change in diastolic tone. If the same concentration of nicotine is introduced simultaneously with excess calcium, the heart stops almost immediately in a relaxed condition. Recovery from this state of diastolic arrest can occur in sea water, in sea water containing nicotine alone, or in sea water containing excess calcium chloride alone. Apparently, excess calcium enhances the action of nicotine (Fig. 8).

j) *The effect of contracture-producing substances on hearts arrested in isosmotic sodium chloride.*—When adrenalin chloride, digitalin, quinine sulphate, or potassium chloride

is added in appropriate concentration to van't Hoff solution, the resulting medium causes prompt and prolonged systolic arrest of the heart through which it is perfused. These compounds, then, may be termed "contracture-producing substances." If, however, one adds one of these substances, in a concentration which would produce contracture if

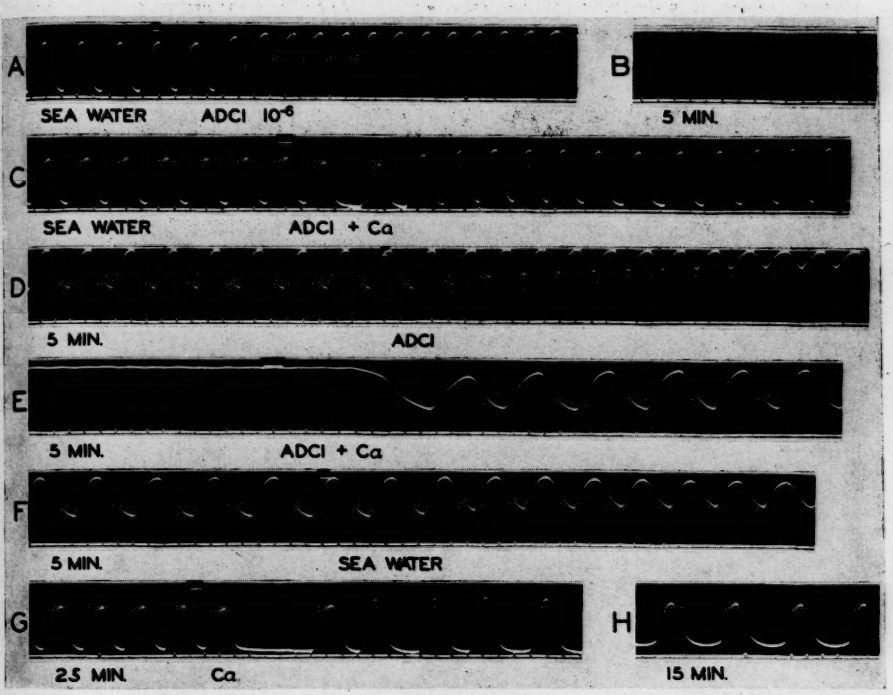

FIG. 6.—The effect of excess calcium on adrenalin action. *A*, at the signal sea water containing adrenalin chloride 1:10⁶ was introduced. *B*, at the end of 5 minutes the heart is in systolic arrest; the shadow of the lever is coincident with the upper margin of the record. *C*, the heart has recovered in sea water; at the signal sea water containing 12 gm. of added calcium chloride per liter and adrenalin chloride 1:10⁶ was introduced. *D*, the same, 5 minutes later; at the signal sea water containing adrenalin chloride 1:10⁶ was introduced. *E*, the same, 5 minutes later; the signal indicates introduction of sea water containing 12 gm. of added calcium chloride per liter and adrenalin chloride 1:10.⁶ *F*, signal indicates return to sea water. *G*, at the signal sea water containing 12 gm. of added calcium chloride per liter was introduced.

added to van't Hoff solution, or even in a greater concentration, to isosmotic sodium chloride and then introduces this medium to a heart which has arrived in diastolic arrest after prolonged perfusion with isosmotic sodium chloride, only a slight and transitory contracture is produced. The records of such an experiment, in which adrenalin chloride was used, are shown in Figure 5. An immediate response is apparent when the adrenalin is introduced, but the perfusion fluid apparently lacks something essential to the maintenance of the contracted state.

k) The effect of contracture-producing substances on hearts arrested in calcium-free van't Hoff solution.—The eventual diastolic arrest resulting when the heart is perfused with van't Hoff lacking calcium has been described above. If one perfuses a heart, thus arrested, with calcium-free van't Hoff solution containing a contracture-producing substance, the result is similar to those described in the previous experiment. Here, again, only a temporary contracture is produced. The results of experiments employing adren-

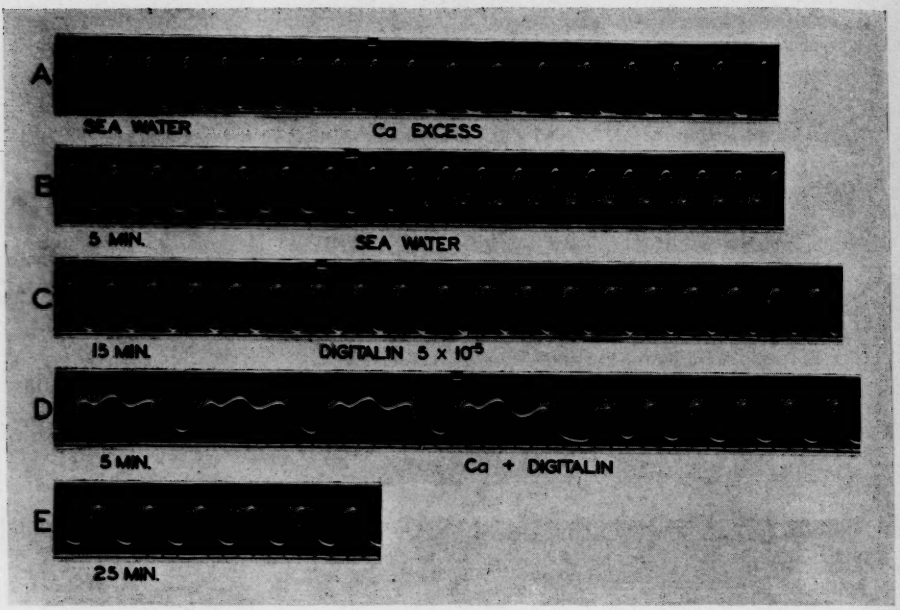

FIG. 7.—The effect of excess calcium on digitalin action. *A*, the signal indicates the introduction of sea water containing 11 gm. of added calcium chloride per liter. *B*, after 5 minutes of perfusion with excess calcium sea water was reintroduced at the signal. *C*, 15 minutes later; digitalin $5:10^5$ introduced at signal. *D*, after 5 minutes of perfusion with digitalin; at the signal sea water containing digitalin $5:10^5$ and 11 gm. of added calcium chloride per liter was introduced. *E*, after 25 minutes of perfusion with digitalin and excess calcium.

alin chloride, quinine sulphate, and digitalin are shown in Figures 9, 10, and 11, respectively. None of these substances can produce a prolonged contracture in a calcium-free solution.

l) The effect of contracture-producing substances in isosmotic calcium chloride.—The results of the experiments just described indicate that the presence of calcium may be essential to the contracture-producing action of several substances. Consequently, several experiments were performed to test the effect of these substances in solutions of isosmotic calcium chloride on hearts which had previously been arrested by prolonged perfusion with isosmotic calcium chloride. Figures 11, 12, 13, and 14 show the records of experiments in which digitalin, adrenalin chloride, quinine sulphate, and potassium chloride

were used. These results show that any of these substances in a solution of calcium chloride can initiate a reversible contracture and maintain it for an extended period.

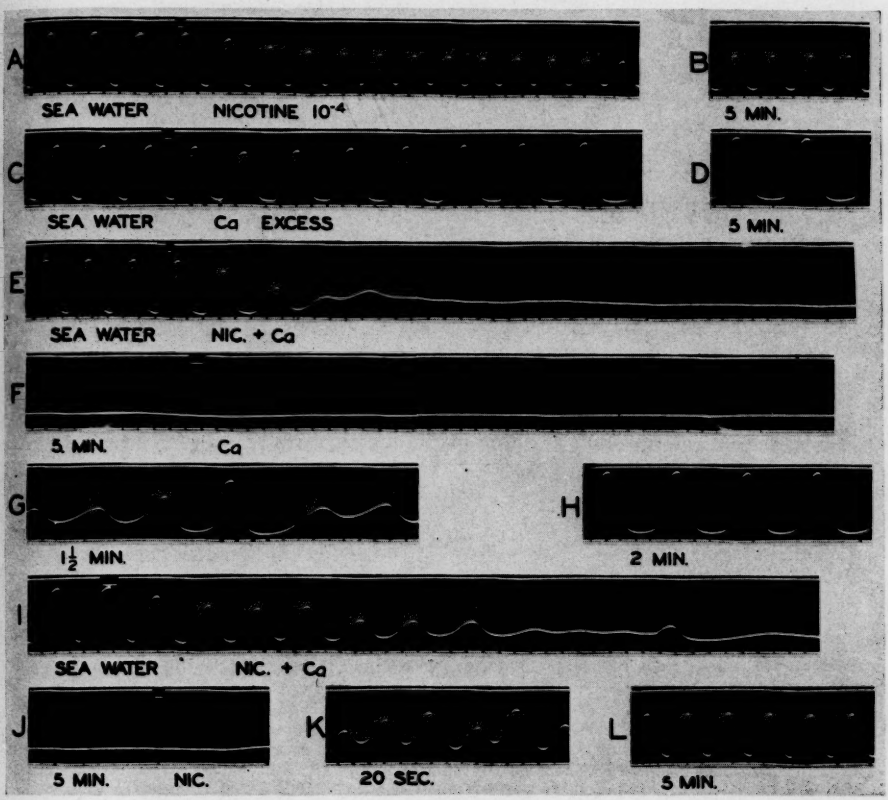

FIG. 8.—The effect of excess calcium on nicotine action. *A*, sea water changed at signal to sea water containing nicotine 1:10,000. *B*, the same, 5 minutes later. *C*, the heart has resumed its normal beat in sea water; at the signal sea water containing 11 gm. of excess calcium chloride per liter was introduced. *D*, the same, 5 minutes later. *E*, the heart has again resumed its normal beat in sea water; at the signal sea water containing 11 gm. of excess calcium chloride per liter and nicotine 1:10,000 was introduced. *F*, the same at the end of 5 minutes; at the signal sea water with 11 gm. of added calcium chloride per liter was introduced. *G*, the same at the end of 1½ minutes. *H*, the same at the end of 2 minutes. *I*, the heart has again resumed its normal beat in sea water; at the signal sea water containing 11 gm. of added calcium chloride per liter and nicotine 1:10,000 was introduced. *J*, the same, 5 minutes later; at the signal sea water containing nicotine 1:10,000 was introduced. *K*, the same, 20 seconds later. *L*, the same at the end of 5 minutes.

m) *The effect of solutions containing calcium chloride plus sodium chloride.*—Mixtures of isosmotic sodium chloride and isosmotic calcium chloride are capable of initiating rhythmic contractions in a heart which has been brought to diastolic arrest in isosmotic

calcium chloride. The ratio of sodium to calcium can be varied over a wide range, as shown in Figure 15. These results seem to point to the importance of the sodium ion in the production of rhythmic action of the heart.

DISCUSSION

These results seem to indicate that the action of contracture-producing drugs on the oyster heart is dependent on the presence of calcium in the perfusion medium; and, since

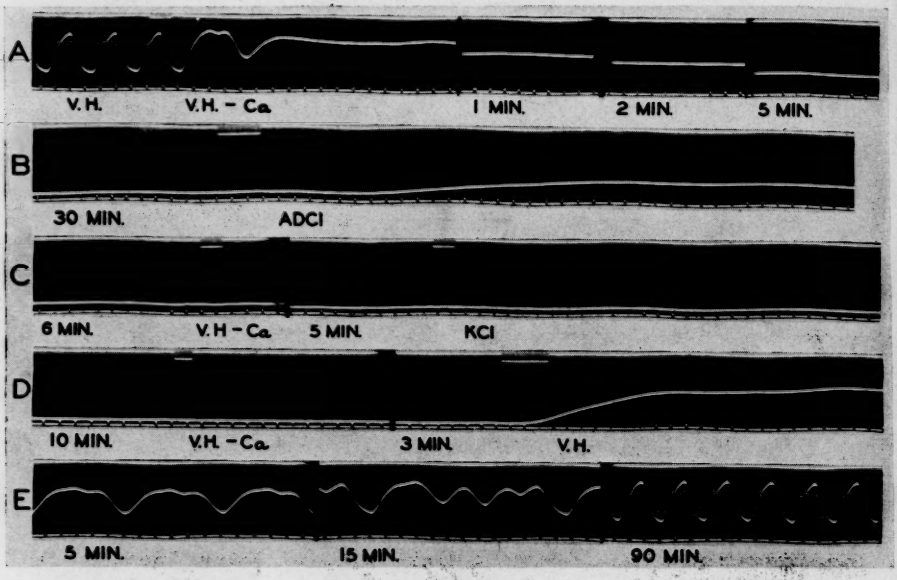

FIG. 9.—The effect of adrenalin chloride in a calcium-free solution and the effect of isosmotic potassium chloride following perfusion with a calcium-free solution. *A*, after the introduction of a calcium-free van't Hoff solution at the signal the heart gradually relaxes. *B*, at the end of 30 minutes the heart is extremely relaxed; calcium-free van't Hoff containing adrenalin chloride $1:10^6$ was introduced at the signal. *C*, at the end of 6 minutes the adrenalin shows no effect; at the first signal calcium-free van't Hoff solution was introduced; after 5 minutes, isosmotic potassium chloride was introduced at the second signal. *D*, at the end of 10 minutes the potassium chloride still shows no effect; at the first signal van't Hoff solution lacking calcium was introduced; after 3 minutes van't Hoff solution was introduced at the signal. *E*, showing recovery in van't Hoff solution.

the stimulating action of sodium and potassium appears also to require the presence of calcium, it seems reasonable to conclude that, in the oyster heart, calcium is primarily involved in the contractile process, as is usually assumed in case of the vertebrate heart.

The dependence of the action of certain drugs on the ionic composition of the medium has been observed by a number of investigators. For example, Burridge (1912) found that an excess of calcium in the perfusion fluid antagonizes the inotropic action of adrenalin on the frog heart. A similar result was obtained, likewise on the frog heart, by Libbrecht (1920), who found, in addition, that, in the absence of calcium chloride, adren-

alin has no effect. Pearce (1913) states that the presence of calcium is necessary for the vasoconstrictor action of adrenalin in perfused frog preparations. Numerous observations of a synergistic action between calcium and the cardiac glucosides might be cited. One which is of particular interest in connection with the present investigation is that of Clark (cited from Bayliss, 1927), demonstrating that the action of digitoxin (one of

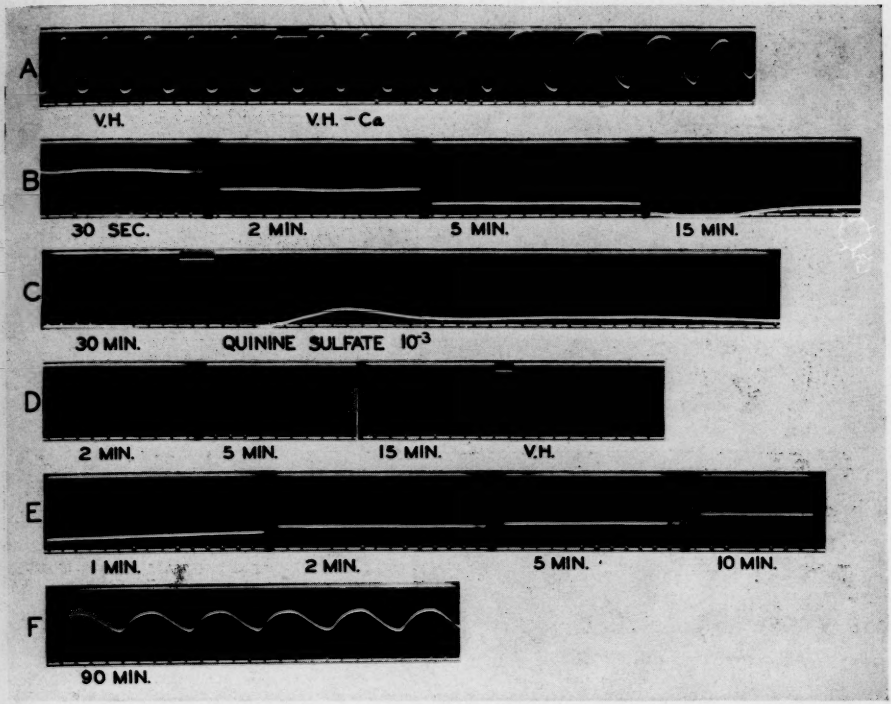

FIG. 10.—The effect of quinine sulphate in a calcium-free solution. *A*, at the signal van't Hoff solution lacking calcium was introduced. *B*, showing the gradual relaxation of the heart in a calcium-free medium. *C*, after 30 minutes the shadow of the recording lever is at the lower edge of the record; at the signal quinine sulphate 1:1,000 in calcium-free van't Hoff was introduced. *D*, the heart is so relaxed that the shadow of the lever is completely off the paper; at the signal van't Hoff solution was introduced. *E*, *F*, showing partial recovery of the heart in the van't Hoff solution.

the active principles of digitalis) on the frog heart is dependent on the presence of calcium in the perfusion medium. The results just noted are all in agreement with the ones obtained on the oyster heart in the present investigation.

The fact that sodium chloride can initiate rhythmic contractions in an oyster heart that has been arrested in calcium chloride is of interest in connection with experiments reported by Baetjer and McDonald (1932). These investigators found that perfusion with sodium chloride produced rhythmic electrical and mechanical beats in the terrapin

sinus that had previously been arrested by continued perfusion with glucose or with calcium chloride.

Recently Heilbrunn (1928–40) has developed a theory which attempts to explain stimulation in terms of colloidal chemistry. The success which he has enjoyed in applying this theory to various vital phenomena warrants its consideration in connection with

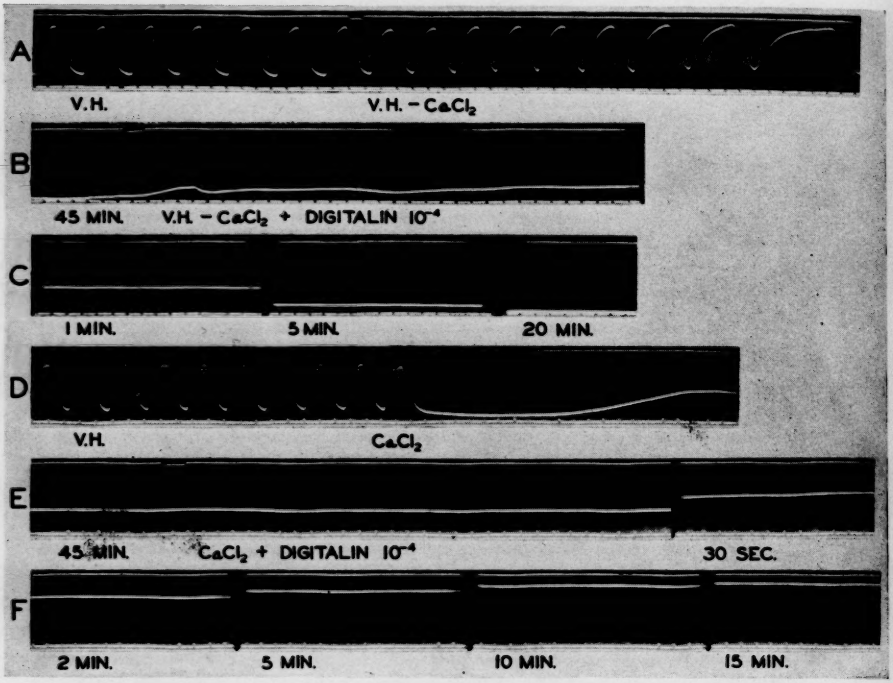

FIG. 11.—The effect of digitalin in a calcium-free solution and in an isosmotic solution of calcium chloride. *A*, at the signal van't Hoff solution was replaced by van't Hoff solution lacking calcium. *B*, at the end of 45 minutes the heart is relaxed to a point lower than normal diastolic level; digitalin 1:10,000 in calcium-free van't Hoff was introduced at the signal. *C*, at the end of 20 minutes the heart is again completely relaxed in spite of the continued presence of digitalin. *D*, after recovery in van't Hoff solution isosmotic calcium chloride was introduced at the signal. *E*, at the end of 45 minutes the heart is in a relaxed state; digitalin 1:10,000 in isosmotic calcium chloride was introduced at the signal. *F*, showing the persistence of the digitalin effect.

the present problem. According to this theory, calcium exists in a bound form, probably as a protein complex, in the cortex of the cell. Stimulation results in the release of the bound calcium to the cell interior, where it takes part in a characteristic clotting reaction.

Heilbrunn (1940*a* and *b*) and Ashkenaz (1940) have applied the above theory to the results of experiments on single fibers from the frog sartorius muscle. If an isolated fiber is immersed in a calcium chloride solution so that the cut ends are in contact with the medium, the fiber shortens rapidly and a coagulation of the interior protoplasm occurs.

This clotting occurs gradually; it begins at the cut ends and proceeds inwardly until the whole inner protoplasm of the cell is coagulated. If only the intact surface of the muscle cell is allowed to come in contact with the calcium chloride solution, no shortening occurs and no coagulum is formed. The fibers will contract, however, if the intact surface is brought in contact with solutions of certain substances, including sodium chloride or

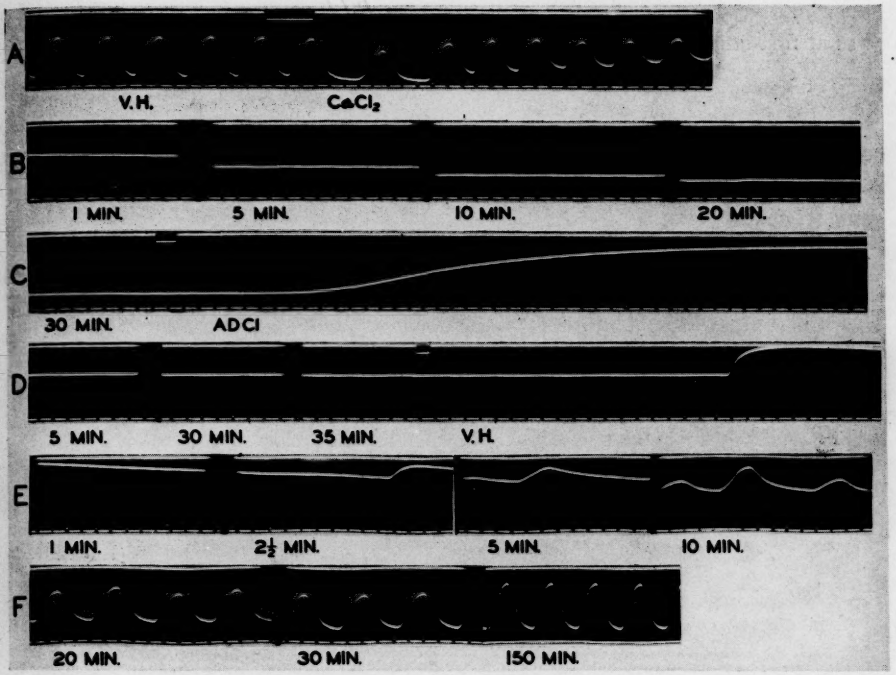

FIG. 12.—The effect of adrenalin chloride in isosmotic calcium chloride solution. *A*, at the signal isosmotic calcium chloride was substituted for van't Hoff solution. *B*, showing temporary contracture followed by gradual relaxation. *C*, after 30 minutes the heart has relaxed to normal diastole; at the signal adrenalin chloride $1:10^5$ in isosmotic calcium chloride was introduced. *D*, at the end of 35 minutes the heart is still contracted to about the normal systolic level; at the signal van't Hoff solution was introduced. *E*, *F*, showing recovery in van't Hoff solution.

potassium chloride. These salts, then, can act as stimulants by releasing the bound calcium of the cortex to the interior of the cell. Evidence for this is shown by the fact that cells which have been immersed for some time in a calcium-free solution do not respond to the stimulating action of potassium or sodium. The presence of excess calcium in a stimulating agent prevents the action of that agent. This is explained by assuming that the presence of excess calcium in the external solution prevents the displacement of calcium from the surface of the cell to the interior.

On the basis of Heilbrunn's theory we may attempt to explain the results obtained on the oyster heart, and presented above, as follows:

a) *The immediate stimulating effect of solutions of sodium and potassium.*—When placed in the presence of excess sodium or potassium, the heart quickly becomes arrested in systole because either sodium or potassium can displace calcium to the cell interior. We may conclude that potassium has a more powerful action than sodium in this respect, since it was seen to produce its effect more rapidly and to cause a more violent contracture.

b) *The effect of continued perfusion with a calcium-free solution.*—The heart, which was at first stimulated to contract by the relative excess of sodium and potassium in

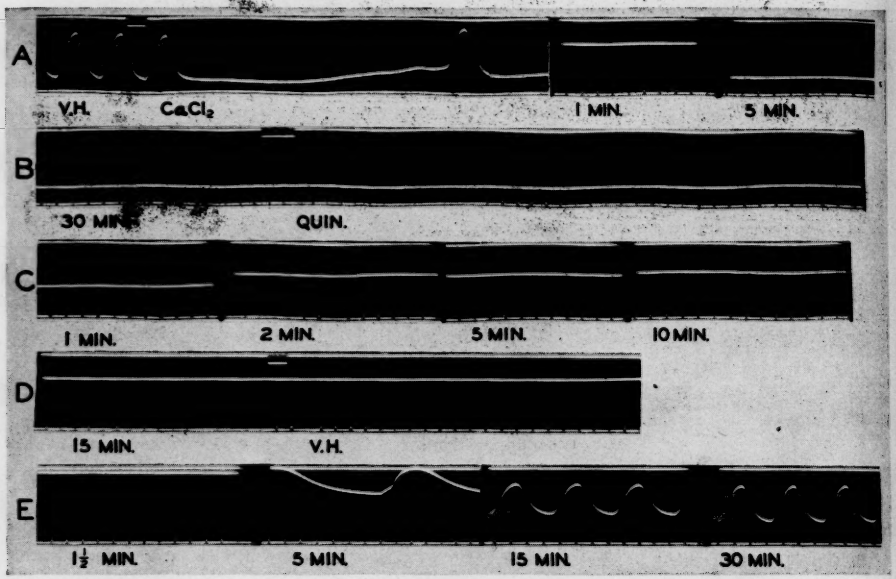

FIG. 13.—The effect of quinine sulphate in isosmotic calcium chloride solution. *A*, at the signal van't Hoff solution was replaced by isosmotic calcium chloride. *B*, at the end of 30 minutes the heart has relaxed to the normal diastolic level; quinine sulphate 1:1,000 in isosmotic calcium chloride was introduced at the signal. *C*, showing the development and persistence of contracture. *D*, at the end of 15 minutes the heart is contracted to about the normal systolic level; van't Hoff solution was introduced at the signal. *E*, showing recovery in van't Hoff solution.

the medium, gradually relaxes, since there is no calcium present in the perfusion medium, and this ion is gradually lost from the cell.

c) *The effect of contracture-producing drugs in the normal perfusion fluid.*—We may assume that a stimulating drug acts by displacing calcium to the interior and that such drugs, since they act in relatively small concentrations, have a more powerful calcium-releasing effect than has sodium or potassium.

d) *The effect of contracture-producing drugs in a calcium-free fluid.*—The slight, transitory contracture produced by these drugs is due to their release of traces of calcium still bound in the cortex of the cell. After this takes place, a permanent relaxation occurs because there is not enough calcium left for continued release.

e) The effect of perfusion with a solution of calcium.—Although there is an abundance of calcium present, this ion is incapable of releasing itself. Since there are no substances present which can cause the release, relaxation occurs. The temporary contracture which occurs is probably due to the presence of calcium-releasing substances which have not yet been washed away. It should be noted that the extent of relaxation in a solution of

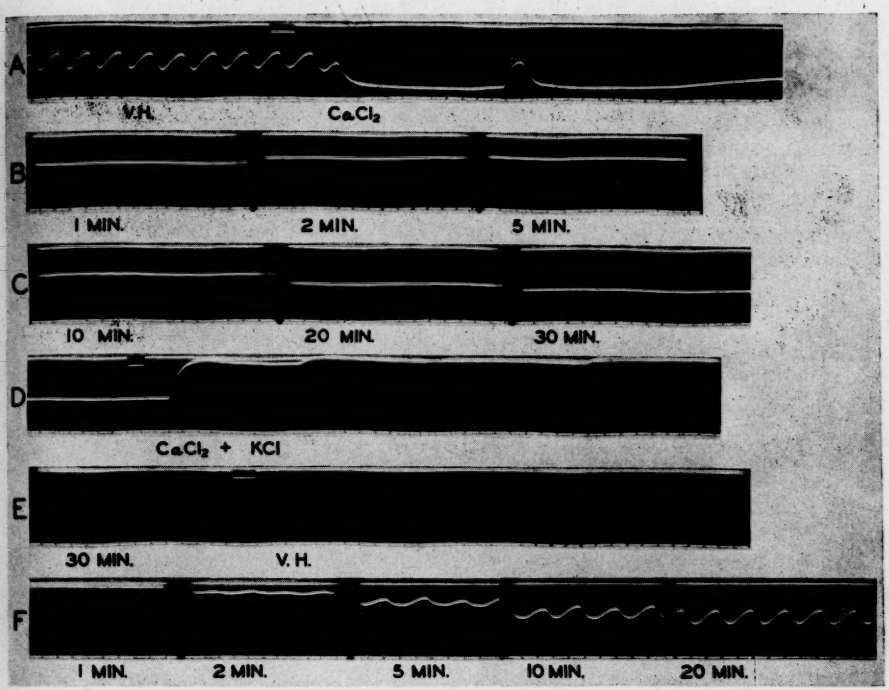

FIG. 14.—The effect of isosmotic calcium chloride and the effect of added potassium chloride. *A*, at the signal van't Hoff solution was replaced by isosmotic calcium chloride. *B*, the heart develops a contracture. *C*, the heart relaxes to the normal diastolic level. *D*, at the signal a solution of equal volumes of isosmotic calcium chloride and isosmotic potassium chloride was introduced. *E*, showing persistence of contracture at the end of 30 minutes; van't Hoff solution was introduced at the signal. *F*, showing recovery in van't Hoff solution.

pure calcium is never so great as that in calcium-free solutions. Under the influence of a steep concentration gradient, calcium may be able to penetrate slowly into the cell and thus maintain a slight degree of contracture.

f) The effect of contracture-producing substances in a solution of calcium chloride.—When the heart is arrested in a relaxed state by continued perfusion with calcium chloride, there is an abundance of bound calcium in the cell, as indicated in the previous paragraph. Since this is continually being renewed by the perfusion fluid, the stimulating substances can produce a prolonged contracture. The effect of mixtures of sodium and

calcium is a special one. About all one can say is that sodium acts in such a way that calcium is released rhythmically rather than continuously.

g) The effect of excess calcium in the perfusion fluid on the action of contracture-producing substances.—It has been mentioned above that calcium acts to prevent its own displacement. Hence, with excess calcium in the solution the cell will have a higher threshold to calcium-releasing substances, i.e., contracture-producing substances.

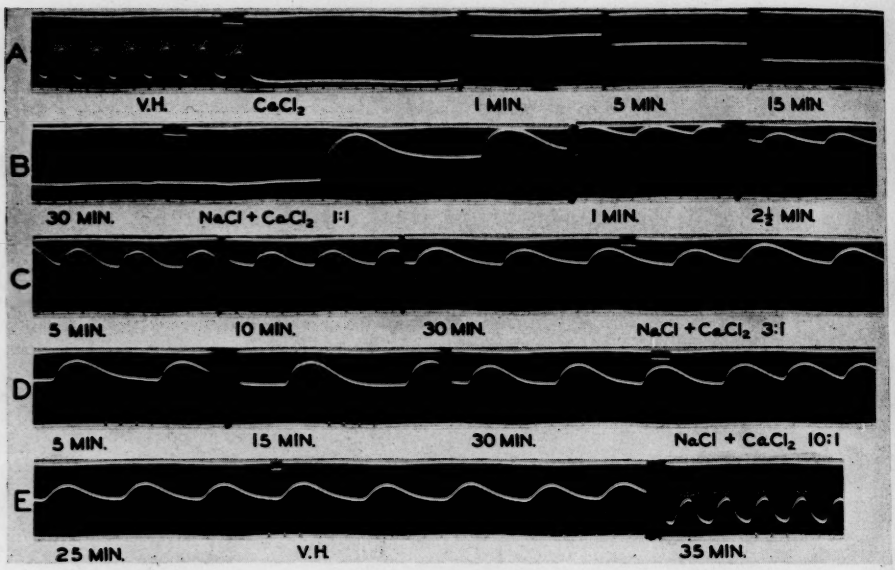

FIG. 15.—The effect of isosmotic calcium chloride plus isosmotic sodium chloride. *A*, at the signal isosmotic calcium chloride was introduced. *B*, after 30 minutes the heart is relaxed to the normal diastolic level; at the signal a mixture of equal parts of isosmotic sodium chloride and isosmotic calcium chloride was introduced. *C*, at the signal a mixture of three parts of isosmotic sodium chloride and one part of isosmotic calcium chloride was introduced. *D*, at the signal a mixture of isosmotic sodium chloride and isosmotic calcium chloride in the ratio 10:1 was introduced. *E*, van't Hoff solution was introduced at the signal.

h) The effect of a depressing substance (nicotine).—It is possible that nicotine may compete with calcium for the substance which normally binds the latter. In the presence of excess calcium, nicotine will be even more effective as a depressing agent, because, as we have already seen, excess calcium seems to reduce the effectiveness of the stimulating ions present.

It appears that Heilbrunn's theory is consistent with the behavior of the cardiac muscle of the oyster as well as with that of frog skeletal muscle (Heilbrunn, 1940*a* and *b*). Obviously, it does not offer as exact an explanation as might be desired, but one must remember that the theory is still incompletely developed. It is hoped that experiments designed to test the applicability of this theory to other types of muscle—vertebrate cardiac muscle, for example—will be forthcoming.

SUMMARY

1. The effect of the hydrogen-ion activity of the perfusion medium on the isolated ventricle of the oyster has been studied. Below pH 4 diastolic arrest occurs. In the range extending from pH 4 to pH 9 the rate of beat increases as the pH is increased. Above pH 9 the beat tends to become irregular, and at about pH 10 the heart stops in a contracted state.

2. The immediate effect of replacing van't Hoff solution with an isosmotic solution of sodium chloride or of potassium chloride as a perfusion fluid for the oyster heart is systolic arrest; the immediate effect of replacement of van't Hoff solution with isosmotic calcium chloride is diastrolic arrest.

3. Continued perfusion with calcium-free van't Hoff solution or with an isosmotic solution of either sodium chloride, potassium chloride, or calcium chloride produces a relaxed condition in the heart; the extent of relaxation is greater in calcium-free van't Hoff solution, or in sodium chloride, or in potassium chloride, than in calcium chloride.

4. Certain substances (adrenalin, quinine sulphate, digitalin, potassium chloride), when added in appropriate concentration to van't Hoff solution, can produce contracture of the heart. When introduced in a calcium-free solution to a heart that has been brought to diastolic arrest in a calcium-free solution, these same substances produce only a slight and transitory effect. If, however, they are introduced in an isosmotic calcium chloride solution to a heart which has been brought to diastolic arrest by continued perfusion with isosmotic calcium chloride, they produce a strong and persistent contracture.

5. These contracture-producing substances are not so effective when introduced in a perfusion medium containing excess calcium chloride as they are in the normal perfusion medium.

6. The depressing action of nicotine on the heart is enhanced by the presence of excess calcium chloride in the perfusion medium.

7. The heart will beat rhythmically in a solution containing only calcium chloride and sodium chloride.

8. The results are discussed in the light of a colloid chemical theory of stimulation.

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THE ACTION OF CUPRIC CHLORIDE ON THE PROTOPLASMIC VISCOSITY OF AMOEBA DUBIA[1]

(One figure)

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THE biological aspect of the "copper problem," as well as the study of all heavy metals, has been attacked experimentally from numerous points of view. Yet, concerning the mechanism of the action of copper, little is actually known. Theories run the gamut from the physiologically unrevealing term "coagulation" to some possible role in catalysis. Undoubtedly, the threshold of a more profound advance in the biological study of heavy metals looms in the application of artificially radioactive elements to this problem.

In perusing the voluminous literature on the action of copper on biological material, it may be stated, generally, that copper in relatively high concentrations is lethal; but in lower concentrations it appears to be stimulative, i.e., produces increased rate of growth, etc. That copper in lethal concentrations expresses itself by coagulating protoplasm, as in single cells, or by precipitating some vital components (enzymes), etc., is a generally accepted conclusion; that copper in very weak concentrations serves as a stimulant to biological material still is debated. This disagreement may express itself not as showing any fundamental dissimilarity in protoplasm but as some induced complication due to a possible removal of copper by physical and chemical binding of the ion either in the cell's external or in its internal medium. (See also Jacobs and Corson, 1934; Angerer, 1937.)

Since copper is known to induce in protoplasm various physiological effects, e.g., increased growth and multiplication, as in yeast (McHargue and Calfee, 1931) and in cardiac explants (Hetherington and Shipp, 1935), and "depressed nuclear growth and division," as in amoebae (Chalkley and Voegtlin, 1932), etc., it is of interest to ascertain the effects of copper on the physical nature of protoplasm itself. Angerer (1937) made a study of the effect of copper on the protoplasmic viscosity of the more "specialized" cell, the unfertilized egg of the sea urchin *Arbacia punctulata* after first calling attention (1933) to the action of high dilutions of copper on the fresh-water protozoan *Amoeba dubia*. The obvious advantage of comparing protoplasm whose normal environment is the sea, which has a high concentration of salts and of precipitants of copper, with protoplasm whose habitat is fresh water is compelling.

METHODS

The material employed in this investigation was *A. dubia;* the specific cytoplasmic layer studied was the more fluid portion, commonly called the "plasmasol."[3] Amoeba

[1] This work was aided by a grant-in-aid from the Society of the Sigma Xi.

[2] This investigation was undertaken originally at the University of Pennsylvania during the year 1931–32, after which it was unavoidably deferred.

[3] For a discussion of the term "plasmasol" as employed here consult Heilbrunn and Daugherty (1932).

cultures were originally purchased from Dr. J. A. Dawson, College of the City of New York, and were cultured in a "balanced" solution. This solution was prepared after the formula of Chalkley (1929) with the exception that the buffer salt, which precipitates Cu^{++}, was omitted. Chalkley's fluid was used as a solvent for all solutions of copper salts and in the preparation of cultures. Some degree of isotonicity was essential, when washing amoebae through various changes of copper in solution, in order to prevent the characteristic polypodality induced by immersion in distilled water.

All salts used in the preparation of solutions were of Merck's "reagent" quality. The solvent for all solutions was obtained from water redistilled from a fused-silica conductivity-still (Kendall).

Chalkley's fluid, after sterilization, was placed in a sterile 4-inch finger bowl to a depth of about ½ inch (*ca.* 100 ml. volume); two sterile grains of wheat and a few drops of fluid from a stock culture of apparently uncontaminated *Chilomonas paramecium* were added. Within 2–3 days, about fifty *A. dubia* were introduced into this culture medium. The cultures were then placed in a cool (17°–21° C.), diffusely lighted room and were not disturbed. After from 3 to 4 weeks the grains of wheat were removed, and the amoebae were tested periodically for "normal" values of viscosity.

The relative viscosity of the plasmasol was determined by the centrifuging method. A Williams, Brown, and Earle (Philadelphia) hand centrifuge, which developed a centrifugal force of one hundred and seventy-seven times gravity when the handle was turned at the rate of one revolution per second, was employed in these experiments. The end-point used, in comparing the centrifuging time of the experimentally treated with the control or "normal" amoebae, was the time required in seconds for a constantly applied centrifugal force to move the intracellular crystals into one-half of the more or less spherical cell. The time in seconds necessary for 80 per cent or slightly more of the amoebae (from eight to twelve amoebae were used for each test) to achieve this end-point is termed the "centrifuging value" or "relative viscosity."

"Normal" amoebae were removed from the stock culture and were washed, generally the night previous to the actual experiment, in several changes of Chalkley's solution in order to remove all extraneous physical and chemical binders of Cu^{++}. The amoebae were allowed to attach themselves to the substratum of a 4-inch Petri dish (*ca.* 60 ml. volume). The supernatant fluid was removed, and the amoebae rapidly covered with the desired concentration of copper. All amoebae immersed in solutions of $CuCl_2$ stronger than 10^{-6} m were studied immediately, while those in the more dilute solutions, including 10^{-6} m, were subjected to two changes of the specific solution of copper. This procedure served to remove the diluting effect occasioned by the presence of the necessary film of water which covered the amoebae after the supernatant fluid had been removed prior to the addition of the solution of copper.

All periods of immersion (abscissae, Fig. 1) were reckoned as the interval of time elapsing from the initial contact of the amoebae with the copper in solution to incipient centrifugalization. Centrifuging values were determined for amoebae immersed for predetermined periods of time in any desired solution of $CuCl_2$ ranging in a series of concentrations from 10^{-3} m to 10^{-12} m. After the general form of the respective curves, indicating the time rate of change in the centrifuging values for the various concentrations of $CuCl_2$, were determined, they were refined further by studying, on a moment-to-moment basis, large batches of amoebae immersed in their respective solutions of copper. Although fewer "positive" centrifuging values were obtained for plotting, these were rein-

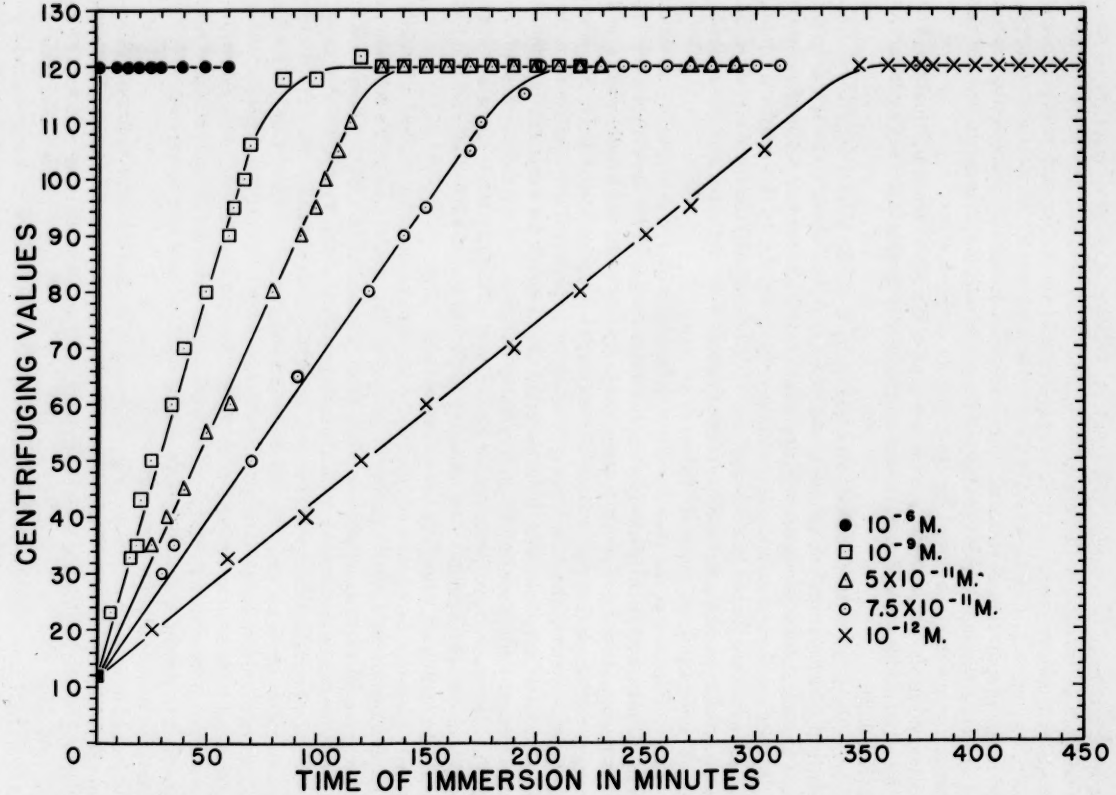

FIG. 1.—Centrifuging value (viscosity) of the plasmasol of *A. dubia* versus time (in minutes) of immersion in solutions of $CuCl_2$ of the designated molarities. Temperature, 19° C.; pH between 6.6 and 6.8.

forced by those "negative" determinations which fell to either side of the rigid value of the end-point employed.

Each curve developed was obtained from amoebae of one stock culture; the remaining amoebae were used to develop a complete viscosity curve for some different concentration of $CuCl_2$. Hence, this procedure afforded a more exact comparison between various curves which represent the action of different concentrations of $CuCl_2$ on the protoplasm of this amoeba.

Finally, in order to remove still further the effect of the variability between cultures and with the previous data as a guide, a set of curves (represented in Fig. 1) was developed in which the amoebae were obtained from one stock culture (see legend under Fig. 1).

All solutions of $CuCl_2$ used and all stock cultures of amoebae were maintained at pH 6.4–6.8. The temperature was fairly constant for any one experiment, but the range of temperature for all experiments was 17°–22° C. All solutions of $CuCl_2$ were prepared freshly, just prior to using, from a m/100 stock solution.

RESULTS

The gross effect produced by a comparison of a series of decreasing concentrations of $CuCl_2$ on the protoplasm of *A. dubia* is that solutions stronger than 10^{-6} m cause an irreversible coagulation, i.e., are lethal, while more dilute concentrations (including 10^{-6} m) give rise to a reversible coagulation, i.e., a gelation.

In so far as the results for the first group of solutions are concerned, the available literature needs no repeating; suffice it to add that, as the lethal effect develops, one observes, invariably, a yellowish-brown wave progressing from the periphery of the rounded amoeba. As a rule, the crystals are moved toward the center of the cell before the oncoming wave, where, finally, they clump and settle under the force of gravity as the cell swells.

The results for the "reversible" action of various concentrations of $CuCl_2$ on the centrifuging values of the plasmasol of *A. dubia* may be obtained from Figure 1. It is found, on determining the "normal" centrifuging value as rapidly as the method permits, that the viscosity of *A. dubia* immersed in 10^{-6} m $CuCl_2$ increases from a "normal "value of 12 arbitrary units to a maximal value of 120 units (900 per cent) within $1\frac{1}{2}$ minutes elapsing from initial immersion to incipient centrifuging. Possibly this increment may be steeper than thus indicated.

When the viscosity is determined on a moment-to-moment basis for *A. dubia* immersed, respectively, in 10^{-9} m, 5×10^{-11} m, 7.5×10^{-11} m, and 10^{-12} m $CuCl_2$, there is found an approximately linear increase in the centrifuging values with time, though, ultimately, the maximal values are approached asymptotically (Fig. 1). These centrifuging values increase from 12 arbitrary units (control value) to a maximum of 120 units. In comparing the different curves, though the respective maxima obtained for the various molarities of $CuCl_2$ are essentially identical, the time rate of approaching these maxima is directly related to the concentration of Cu^{++} present in the bathing medium. The maximal centrifuging values obtained are not strictly identical, as so fortuitously represented for the particular culture of amoebae studied. Values obtained for a specific culture are relatively identical when allowance is made for inherent experimental error. A more pronounced variation occurs in comparing maximal centrifuging values of experi-

mentally treated amoebae from different cultures. The average maxima for all experiments is of the order 110 ± 10 arbitrary units, though the slope of the respective curves is more congruent.

All maximal values are maintained for an indefinite interval of hours, generally overnight (*ca.* 12–15 hours), after which they tend to decrease to a value nearer the original maximum than to that of the controls. This "wearing-off" effect is characteristic.

When copper-treated amoebae of this "reversible gelating series" are repeatedly washed with and replaced in Chalkley's fluid, i.e., in a "normal" environment, the centrifuging values progressively decrease until, finally, the control value is attained.

An interesting observation, in this direction, occurred during the preliminary experiments when spring water (Great Bear Spring Water Company) was being considered as an "isotonic" culture medium. It was observed, on using this fluid for the preparation of 10^{-6} m $CuCl_2$, that the maximal experimental value was obtained; but subsequently, on continuing the tests to determine the maintenance of this value, it was found that it had decreased steadily, within a period of minutes, to that value characteristic of the control. Although no visible precipitation was present, a larger volume of this solution, when permitted to stand approximately for a day, showed a detectable precipitate on the bottom of the container. This condition could be simulated when some precipitant of copper, e.g., bicarbonate, was added to the fluid containing the copper-treated amoebae.

DISCUSSION

Angerer (1937), in studying the protoplasmic viscosity of the unfertilized eggs of *Arbacia punctulata*, showed, indirectly, an apparent surface effect during a so-called "latent period"; at the end of this time the addition of some remover of Cu^{++}, e.g., washing the eggs in sea water, would fail to produce the anticipated change in viscosity and permit the fertilization of the eggs on the addition of spermatozoa. In *A. dubia* the surface effect is more manifest. In favor of this is the sudden increment (an increase of ten times within 1½ minutes or possibly less) in the centrifuging value, during which time copper hardly would have permeated the membrane; furthermore, this effect produced by copper is lost in almost the same interval of time by the addition of some precipitant, e.g., bicarbonate. Jacobs and Corson (1934) have shown indirectly a surface effect in that an inhibition of hemolysis occurred on the addition of a trace of copper, but this protection was removed on the addition of more erythrocytes or some absorbent, e.g., charcoal.

It is obvious that with longer times of immersion copper must enter the cell, at least, in order to produce irreversible coagulation. On observing the hyaline area of the centripetal pole of either a centrifuged *A. dubia*, *A. proteus*, or sea-urchin egg, one can readily observe fine "granules" making their appearance *de novo*. This effect could not be observed in *A. dubia* on using a "reversible gelating concentration" of copper. Immersing copper-treated amoebae in nonlethal concentration of sodium diethyl dithiocarbamate or potassium ethyl xanthate failed to give the characteristic color test within the cell. Reznikoff (1926), on comparing the refractive index of copper-treated with "normal" *A. proteus*, concluded that at least a small amount of copper entered the cell. Chalkley and Voegtlin (1932), who studied *A. proteus* in various concentrations of copper, found that nuclear growth and division were inhibited, thus indicating a possible penetration of copper into the cell.

The action of high dilutions of copper was shown by von Nägeli (1893), who maintained that concentrations as low as 10^{-42} had a deleterious effect on algae.[4] Cramer (1893) found that the lethal concentration for *Spirogyra* was 10^{-6} ($CuSO_4$), while Dreschel (1921) showed that the deleterious effect could be obtained at a concentration of 10^{-8}. The lethal concentration for *A. dubia* lies close to 10^{-6} m $CuCl_2$; but there is a dilution, including this molarity, in which copper is physiologically potent. The progressive increase in viscosity with time (Fig. 1) can be explained by considering the number of copper ions colliding per unit of time with a unit area of cell's surface; of course, the collision rate is a function of the concentration of the ionic species. The limiting or maximal centrifuging values render themselves less easily to interpretation. Is it that these dilutions (Fig. 1) are without deleterious result, owing to the cell's ability, biologically, to incorporate the ion into its system, but that, in so doing, Cu^{++} expresses itself physiologically by a reversible gelation of the protoplasm? This change in protoplasmic viscosity is reflected in the "proteus"-like character of locomotion which the copper-treated *A. dubia* simulates. When "normal" *A. dubia* are centrifuged for 1 second in a high-speed centrifuge (4,084 times gravity), the cells erupt. However, on centrifuging *A. proteus* simultaneously with a fully copper-treated *A. dubia*, the intracellular crystals are moved centrifugally at approximately the same time rate. This fact is reinforced by centrifuging the crystals to the centrifugal pole in order to measure the subsequent width of the plasmagel layer. When comparing the copper-treated *A. dubia* with the "normals," it was apparent that the plasmagel of the former layer was of greater width.

SUMMARY

1. The effect of various concentrations (10^{-3} m to 10^{-12} m) of $CuCl_2$ on the viscosity of the plasmasol of *A. dubia* was determined by the centrifuging method.

2. Categorically, the action of high concentrations of Cu^{++} on the protoplasm of *A. dubia* produces an irreversible coagulation, i.e., is lethal (concentrations stronger than 10^{-6} m $CuCl_2$); whereas more dilute solutions (10^{-6} m and weaker) cause a reversible coagulation, i.e., a gelation.

3. A study of those solutions of $CuCl_2$ which give the reversible gelating effect (see Fig. 1) show that the respective centrifuging values (relative viscosity) increase linearly but ultimately approach their maxima asymptotically. This increase is of the order of 900 per cent. The time rate of approaching the maxima for the respective molarities of $CuCl_2$ is directly related to the concentration of $CuCl_2$ present in the immersing medium.

4. Any substance tending to precipitate or bind the Cu^{++} removes this effect of copper. It is suggested that Cu^{++} initially exerts its physiological effect at the cell's surface and that subsequent penetration is of secondary importance.

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REVERSAL OF CILIARY ACTION IN PARAMECIUM INDUCED BY CHEMICALS

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CILIARY activity has been the subject of many investigations, partly for its intrinsic value but largely in the hope that it would lead to an understanding of the general phenomena of protoplasmic contractility. It has long been held (Hartmann, 1933; Gray, 1928, 1931) that the phenomena exhibited in ciliary activity are of essentially the same order as those exhibited by muscular tissue and pseudopodial movement and that a theory which will adequately explain one will doubtless be applicable to the others.

Reversal in the direction of the effective stroke of the cilia (lasting from a few seconds to several minutes) induced by increase in concentration of certain chemicals in the environment is very common in the Protozoa. Ciliary reversal has also been reported in many of the Metazoa, and the reproductive cells of animals and plants have been observed to swim backward; however, physiological and environmental factors involved in the phenomenon are little understood.

Jennings (1899, p. 319) noted that in paramecia put into a 1 per cent solution of KI the action of the cilia reverses for 6–8 minutes. Merton (1923) obtained reversal of ciliary action in *Stentor coeruleus* in solutions containing sodium and potassium ions but did not obtain reversal in solutions of $CaCl_2$, $MgSO_4$, and $MgCl_2$. He found (1935) in further studies on *Stentor* and *Stylonychia* that a number of salts of potassium, sodium, rubidium, cesium, and ammonium induce reversal in the action of cilia. Reversal, however, was not obtained with calcium salts. He says that reversal results from some specific action of the alkali metals, though he does not say what the specific action is. Oliphant (1938) found that the chlorides, iodides, and bromides of potassium, lithium, sodium, and ammonium induce reversal in the direction of the effective beat of the cilia of *Paramecium* and that the chlorides, iodides, and bromides of calcium and magnesium do not. He believes that ciliary reversal is due to the action of the monovalent cations and suggests that it is associated with an increase in protoplasmic viscosity. Peter (1899) was the first to show, and Alverdes (1922), Worley (1934), Horton (1935), and others have confirmed and extended the observation, that in fragments of ciliated protozoans the cilia continue to beat as long as they are connected with a fragment of cytoplasm. Such ciliated fragments have also been observed to reverse direction of ciliary beat in response to environmental change. This suggests that cilia are automatic in their movement and that inhibition or variation in ciliary activity is of an extraneous nature.

The only survey to explore what classes of chemicals induce reversal in ciliary action is a preliminary one by Mast and Nadler (1926), who studied *Paramecium caudatum*. They investigated fifty-six different chemicals—all but eleven of them salts or hydrates of the alkali metals or alkaline earths. They found that all the univalent cation salts and hydrates tested induced ciliary reversal except $(NH_4)_2SO_4$ and $NH_2 \cdot C_2 \cdot H_3O_2$ but none of the bi- and trivalent cation salts and hydrates except $CaHPO_4$, $MgHPO_4$, and

$Ba(OH)_2$ .They obtained reversal in phosphoric and oxalic acids but not in CHl. These were the only acids tested. Ciliary reversal was not obtained in solutions of lactose, the only nonelectrolyte tested. They conclude (p. 117): "Ciliary reversal is associated with differential absorption of the cations and subsequent changes in electric potential, but there are also other factors involved."

The data of Mast and Nadler are incomplete, and only in the case of eighteen chemicals do they give any indication of the range of concentrations used and other conditions of the tests, and only for the chlorides of sodium, potassium, lithium, and ammonium do they indicate the magnitude of the response. In view of the work of Merton and Oliphant the exceptions cited by Mast and Nadler are difficult to explain; and, if the chemicophysical factors in ciliary reversal are to be ascertained, there is indicated the desirability for a further survey to check these exceptions and to find whether there are other chemicals or classes of chemicals which induce reversal in ciliary action. It is the object of the present investigation to make such a survey with the view of ascertaining the relation between various chemicals in the environment and reversal in ciliary action and contributing toward the analysis of the chemicophysical factors involved in protoplasmic contractility.

MATERIALS AND METHODS

Paramecium multimicronucleatum Powers and Mitchell was used in this investigation. The specimens were obtained from a clone isolated from a strain which has been grown in the laboratory at Stanford for more than 10 years. The organisms were cultured on an infusion consisting of 0.2 gm. of dehydrated leaf meal of alfalfa in a liter of distilled water. This infusion was autoclaved at 15 pounds for 20 minutes and poured into 200-cc. Erlenmeyer flasks. After 24 hours, 5 cc. of old infusion containing numerous paramecia were added to each flask. New cultures were prepared each week, and no culture was used for experimentation until after it had stood for a week. By this time the hydrogen-ion concentration of the culture was pH 6.8 ± 0.1. The cultures were kept in an incubator at 23° C. No special effort was made to control the bacterial flora; however, by this method animals in nearly the same physiological condition were obtained for all of the experiments.

The chemicals used were, with few exceptions, Merck's blue label reagents and were all of high purity. Chemical solutions were prepared with triple-distilled water.

In making the tests paramecia were first thoroughly washed in 3× balanced salt solution (Barker and Taylor, 1931), transferred to pure wash solution, and left for an hour before being used for experimentation. To ascertain the effect of chemicals on ciliary reversal, 2.5 cc. of the solution to be tested were put into a modified Columbia culture dish on the stage of a binocular dissecting microscope. One of the washed paramecia was taken up in a capillary pipette and put into the test solution. The specimen was observed continuously (28×) until the end of the experiment; and, if ciliary reversal occurred, its duration was measured with a stop watch. This was repeated with other specimens as often as was desired. Ciliary reversal was recorded only if the animals actually swam backward for a time readily measurable with a stop watch.

EXPERIMENTAL RESULTS

The results of an extensive series of experiments are summarized in Table 1. In the first column of the table are listed the different chemicals tested. The second column

TABLE 1

THE EFFECT OF INCREASE IN CONCENTRATION OF VARIOUS CHEMICALS IN THE ENVIRONMENT ON REVERSAL IN CILIARY ACTION IN *Paramecium*

Substance Tested	Range of Concentrations Tested*	Ciliary Response†
KCl	M/1 –M/1,000	Reversal 0.75–2.0 minutes in optimum higher concentrations
KBr	M/1 –M/1,000	Reversal 0.75–2.0 +
KI	M/1 –M/1,000	
KNO_3	M/5 –M/1,000	Reversal 0.5 –1.5
K_2CO_3	M/20 –M/2,500	Reversal 1.0 –2.0
KCN	M/10 –M/10,000	Reversal 0.75–2.5
KOH	M/50 –M/5,000	Reversal 0.75–2.0
NaCl	M/1 –M/1,000	
NaBr	M/1 –M/1,000	Reversal 10–25 seconds
NaI	M/1 –M/1,000	
NaCN	M/10 –M/2,500	
$NaNO_3$	M/1 –M/1,000	Reversal 10–15
$NaC_2H_3O_2$	M/1 –M/1,500	Reversal 3– 8
$NaNO_2$	M/1 –M/1,000	Reversal 5–10
Na_2CO_3	M/5 –M/250	Reversal 5– 8
NaOH	M/50 –M/2,500	Reversal 25–40
LiCl	M/1 –M/1,000	Reversal 0.75–2.0+ minutes
LiBr	M/1 –M/1,000	
LiI	M/1 –M/1,000	Reversal 1.0–2.5
Li_2CO_3	M/1 –M/1,000	Reversal 0.5–1.5
NH_4Cl	M/5 –M/200	Reversal 15–30 seconds
NH_4Br	M/5 –M/200	
NH_4I	M/20 –M/300	
$(NH_4)_2SO_4$	M/5 –M/200	Reversal 10–15
$NH_4C_2H_3O_2$	M/5 –M/200	
NH_4NO_3	M/10 –M/200	
NH_4OH	0.002% –0.00001%	Reversal 35–60
$BaCl_2$	M/250 –M/500,000	Reversal 1.0–2.5 minutes
$Ba(OH)_2$	M/300 –M/500,000	Reversal 1.0–3.0
$MnCl_2$	M/20 –M/5,000	Reversal 0.75–1.75
$MnSO_4$	M/20 –M/5,000	
Oxalic acid	0.1% –0.0001%	Reversal 1.5–3.0 seconds
H_2CO_3	Saturated solution	Reversal 30–60 seconds and longer after prolonged exposure
$CaCl_2$	M/10 –M/10,000	No reversal in any concentration, marked shock reactions
$CaBr_2$	M/10 –M/10,000	
CaI	Dilutions from saturated solution	
$Ca(OH)_2$	Suspension (saturated solution)	
$CaHPO_4 \cdot 2H_2O$	M/900 –M/10,000	
$MgCl_2$	M/10 –M/10,000	
$MgBr_2$	M/10 –M/10,000	
MgI_2	Dilutions from saturated solution	

* Range of concentrations tested does not, in solutions inducing ciliary reversal, represent range from lowest immediately lethal to no reaction, though in most instances it rather closely approximates this range.

† Times are for duration of initial period of reversed activity. This is often followed by recovery and subsequent reversals. Range, rather than averages, are indicated.

TABLE 1—*Continued*

Substance Tested	Range of Concentrations Tested*		Ciliary Response†
$MgHPO_4 \cdot 7H_2O$	M/250	–M/10,000	No reversal as defined here but pronounced avoiding reactions
$SrCl_2$	M/10	–M/5,000	No reversal but a series of avoiding reactions extending over a long time
$ZnCl_2$	M/1,500	–M/10,000	Reversal before death in concentrations lethal in few seconds; no reversal in other concentrations
$ZnSO_4$	M/1,500	–M/10,000	
$CuCl_2$	M/2,000	–M/100,000	Reversal before death in concentrations lethal in few seconds; no reversal in other concentrations (not as marked as in zinc salts)
$CuSO_4$	M/2,000	–M/100,000	Reversal before death in concentrations lethal in few seconds; no reversal in other concentrations
$SnCl_2$	M/1,000	–M/100,000	Typically no reversal in any concentration
$HgCl_2$	M/40,000	–M/500,000	Typically no reversal in any concentration; sometimes reverse before death in high concentrations
$NiSO_4$	M/300	–M/10,000	Typically no reversal in any concentration; sometimes reverse before death in high concentrations
$CO(NO_3)_2 \cdot 6H_2O$	M/250	–M/10,000	No reversal but marked avoiding reactions; may some times reverse just before death in higher concentrations
$UO_2NO_2 \cdot 6H_2O$	M/10,000	–M/200,000	
$AlCl_3$	M/7,500	–M/100,000	
$FeCl_3$	M/15,000	–M/200,000	
$FeSO_4 \cdot 7H_2O$	M/10,000	–M/200,000	
HCl	0.1%	–0.0001%	Marked avoiding reactions but no reversal as defined here
HNO_3	0.1%	–0.0001%	
H_2SO_4	0.1%	–0.0001%	
H_3PO_4	0.1%	–0.0001%	
Acetic acid	0.1%	–0.0001%	
Lactic acid	0.1%	–0.0001%	
Propionic acid	0.1%	–0.0001%	
Maleic acid	0.1%	–0.0001%	
Citric acid	0.1%	–0.0001%	
Tartaric acid	0.1%	–0.0001%	
Picric acid	0.1%	–0.0001%	
Phenol	M/100	–M/5,000	
Formalin	2.0%	–0.001%	
2–4 dinitrophenol	M/2,500	–M/100,000	No reversal in any concentration; typically great increase in rate of forward movement
Urethane	M/5	–M/1,000	No reversal in any concentration
Methyl alcohol	14.0%	–0.001%	No reversal in any concentration; typically great increase in rate of forward movement
Ethyl alcohol	8.0%	–0.001%	
Iso-propyl alcohol	5.0%	–0.001%	
N butyl alcohol	2.0%	–0.001%	
Benzyl alcohol	1.0%	–0.001%	
Ethyl ether	10%	–0.001%	
Acetone	15.0%	–0.01%	
Glucose	M/1	–M/5,000	Usually reversal before death in very high concentrations; typically no reversal in any concentration not lethal in a few seconds
Sucrose	M/1	–M/5,000	
Urea	M/1	–M/1,000	
Lactose	M/5	–M/5,000	Marked avoiding reactions in very high concentrations; typically no reversal in any concentration

shows the range of concentrations over which the chemicals were tested. It is not practicable or particularly instructive in such a table to list each concentration of each chemical investigated. An effort was made to test concentrations from those somewhat higher

than the lowest immediately lethal to those of such dilution as would result in no or only a very weak shock or avoiding reaction. Enough concentrations were tested between these extremes to make reasonably certain that those concentrations inducing reversal in ciliary action were included. As examples, KCl, NaCl, LiCl, and most of the monovalent cation salts were tested in M/1, M/5, M/10, M/20, M/50, M/100, M/200, M/500, and M/1,000 concentration. $Ba(OH)_2$ was tested in M/250, M/500, M/1,000, M/2,500, M/5,000, M/10,000, M/50,000, M/100,000, M/250,000, and M/500,000. The mineral acids were tested in 1.0, 0.1, 0.01, 0.001, and 0.0001 per cent; the organic acids, in twice this number of concentrations over the same range. In the third column of the table is given the ciliary response to each chemical tested. It was observed that the behavior was not the same in all instances, even in those solutions which induce reversal in the action of the cilia. For example, in the higher concentrations of almost all the monovalent cation salts there was an initial period of reversed ciliary action of 1.5–3.0 minutes' duration, which was followed by momentary recovery and normal forward movement, after which there were repeated reversals of several seconds and recoveries until death. The duration of the initial period of reversed ciliary action varied greatly in different salts. In most of the hydrates inducing reversal there was at first a shock reaction in which the animals revolved in a plane about the posterior end without rotating on the longitudinal axis. This continued in the case of $Ba(OH)_2$ for 10–15 seconds, after which the animals swam backward for 1.5–2.5 minutes. This initial reversal was followed by recovery and normal forward movement, after which there were subsequent reversals and recoveries, extending in some concentrations for over half an hour. This type of behavior, though less in degree, was also noted in $BaCl_2$, $MnCl_2$, and $MnSO_4$. In acetic acid and a number of organic acids, as well as in some other substances, as indicated in the table, there were violent avoiding reactions in which the reversed phase averaged almost a second; however, in no instance was this avoiding reaction considered as ciliary reversal, as defined here. In many of the solutions, particularly the nonelectrolytes, there were pronounced shock reactions but no avoiding reactions and no reversal. In $SrCl_2$ the animals swam forward several seconds, hesitated, stopped, swam backward for a fraction of a second, then resumed forward movement, and continued this behavior for several hours. This is the "Ruckwartsbewegungreaktion" described by Eisenberg-Hamburg (1932) and is not considered here as ciliary reversal. Immediately before death in solutions of salts of many of the heavy metals (particularly when time to death was less than a minute) the animals began to swim backward and continued to do so until they died. For all chemicals inducing it, duration of reversed ciliary action varied directly with concentration. It is not possible in Table 1 to summarize the detailed response in each solution, and the third column is intended only to indicate qualitatively the nature of the ciliary response.

Table 1 shows that increase in concentration of all the monovalent cation salts and hydrates tested induces reversal in the direction of the effective stroke of the cilia in *Paramecium*. It shows, too, that the hydrates of the alkali metals are more effective in inducing the response than are the salts. Mast and Nadler say (1926, p. 107) that they obtained reversal in all the monovalent cation salts tested except $(NH_4)_2SO_4$ and $NH_4C_2H_3O_2$. Table 1 shows that reversal in ciliary action is induced by solutions of ammonium sulphate and acetate under the conditions of the present investigation. The average duration of reversal for ten specimens in M/5 $(NH_4)_2SO_4$ was 13.21 ± 1.81 seconds and for ten specimens in M/10 $NH_4C_2H_3O_2$ was 11.24 ± 2.23 seconds. It is possible that difference in results of the two investigations may have been due to differences in

physiological states of the organisms used traceable to conditions in the infusions in which they were grown or in the wash solutions to which they were adapted. That the differences were not due to the different species of *Paramecium* used was established by the fact that results obtained on *P. caudatum* under the conditions of the present investigation are essentially the same as those which obtain for *P. multimicronucleatum*. The action of $(NH_4)_2SO_4$ and $NH_4C_2H_3O_2$ is therefore similar to that of all other ammonium salts tested.

Table 1 shows further that increase in concentration in the environment of none of the bi- and trivalent cation salts and hydrates tested induce ciliary reversal except $BaCl_2$, $Ba(OH)_2$, $MnCl_2$, and $MnSO_4$. $BaCl_2$ and $Ba(OH)_2$ are shown in the table to be far more effective than any other substances tested in inducing reversal, being effective in dilutions of M/250,000–M/500,000. Why this should be when salts and hydrates of other alkaline earths fail to induce reversal is not clear. It is well established, however, and it is reflected in the table, that, unlike the other elements of its group (calcium and strontium), barium is exceedingly toxic. It has been shown (Sollmann, 1932) that salts of barium, in contrast with those of calcium and strontium, stimulate all types of muscle to violent contraction regardless of innervation and stimulate violent peristalsis, culminating in spasm. It seems, therefore, not unreasonable to assume that the action of barium compounds is due to some specific biological action of the barium not shared, at least in degree, by the other elements of its group. Mast and Nadler obtained ciliary reversal with $Ba(OH)_2$ but not with solutions of $BaCl_2$. This failure is very difficult to understand. Under the conditions of the present investigation $BaCl_2$ was found to be second only to $Ba(OH)_2$ in effectiveness in inducing reversal, and in M/500–M/1,000 solutions paramecia may swim backward up to 3 minutes without interruptions, and in M/100,000 the initial period of reversed ciliary action was 4.2 ± 1.23 seconds. Ciliary reversal was observed in $MnCl_2$ and $MnSO_4$ from M/50 to M/10,000 lasting 1.0–2.0 minutes or longer in the higher concentrations, though the initial reversal was often short, followed by recovery and subsequent reversals. It is possible that this action of manganese is attributable to some specific biological action not shared with other elements of its group. However, the salts used in the tests contained, among impurities, for the chloride 0.25, and for the sulfate 2.5, per cent unspecified alkali and earths; and it might be questionable whether the ciliary reversal was due to manganese or impurities.

Mast and Nadler (1926, p. 107) say, regarding bi- and trivalent cation salts and hydrates tested, that none except $CaHPO_4$, $MgHPO_4$, and $Ba(OH)_2$ induced reversal. Table 1 shows that, as in other calcium and magnesium compounds, ciliary reversal was not obtained in a solution of $CaHPO_4 \cdot 2H_2O$ or of $MgHPO_4 \cdot 7H_2O$. The phosphates considered are difficult to use or control in biological work; and their action is affected by extrinsic factors, such as age of the salt, H-ion concentration of the solvent, temperature, and impurities. It is possible that the reversal observed by Mast and Nadler was due to impurities or other extrinsic factors; however, a comparison of results of the two investigations may be equivocal.

It is shown further in Table 1 that increase in concentration in the environment of mineral and organic acids does not induce ciliary reversal in *Paramecium* except in solutions of oxalic acid and, under certain conditions, H_2CO_3. The duration of reversal in 0.01–0.001 per cent oxalic acid was 2.5 ± 0.5 seconds, and the initial reversal was often followed by recovery and normal forward movement, then by subsequent reversals. Why ciliary reversal should be induced by oxalic acid and not by closely related organic

acids is not clear. Oxalate ions have been shown to prevent the clotting of blood through the removal of free calcium, and Heilbrunn (1937) and his students have shown that removal of free calcium through presence of the oxalate ion prevents the surface precipitation reaction. It may be that ciliary reversal in solutions of oxalic acid is due to the action of monovalent cations freed from the antagonistic action of calcium ions when the calcium is bound. Citric and tartaric acids would not be expected to produce similar results because their calcium salts are much more soluble than is calcium oxalate.

In the experiments with H_2CO_3 the organisms were placed in a modified Englemann gas chamber through which carbon dioxide from a Kipp generator was bubbled. The animals reacted at first to the increased H_2CO_3 by numerous shock and slight avoiding reactions and by a marked increase in rate of forward locomotion. This behavior continued normally 7–10 minutes, after which the rate of movement slowed appreciably and reactions became "sluggish." The animals also became more darkly granular in appearance. At the end of about 12 minutes the organisms began to swim backward and usually continued to swim backward until death, which resulted in 1.5–3.0 minutes, though in some cases the initial reversed period was followed by momentary recovery and subsequent reversals.

Table 1 also shows that ciliary reversal is not induced (typically) by increase in the environment of any nonelectrolyte tested. In concentrations of glucose, sucrose, and to a lesser degree urea, which were lethal in a few seconds (as, for example, M/1), many organisms, as they died, swam backward for periods up to 5–10 seconds. In higher concentration of lactose there were marked avoiding reactions which extended over relatively long periods. Typically in nonelectrolytes, as in more dilute solutions of those above and in the alcohols, acetone, and ether, there was marked increase in the rate of forward movement, with sudden changes in direction of locomotion but no avoiding reactions and nothing which could be considered ciliary reversal.

Ciliary reversal in high concentrations of glucose, sucrose, and urea are undoubtedly associated with osmotic phenomena; however, it is obvious from Table 1 that ciliary reversal cannot be directly associated with the osmotic values of the solutions inducing it. If it were, reversal times of the same magnitude would be expected for any given concentration of neutral monovalent cation salt tested, as was pointed out by Mast and Nadler. Moreover, if reversal were directly associated with the osmotic values of the solutions, isosmotic solutions of bi- and trivalent cation salts, as well as nonelectrolytes, would be expected to induce the reaction. Alverdes (1922) and others have induced ciliary reversal in many organisms by increasing the viscosity in the culture medium with quince-seed jelly; and it may be that the reversal observed in glucose, sucrose, and urea was induced by the increased viscosity of the solution. It is also shown in Table 1 that ciliary reversal occurs in both alkaline and acid solutions. However, it is also quite apparent from the table that, if ciliary reversal is dependent upon the H-ion concentration of the medium, it certainly is not directly associated with it, for, while oxalic and carbonic acids induce ciliary reversal, other acids tested over a similar range of H-ion concentrations do not; and this is likewise true for NaOH, KOH, and NH_4OH, as contrasted with $Ca(OH)_2$, and for potassium, sodium, and lithium salts, as contrasted with those of NH_4. Moreover, ciliary reversal is induced by various chemicals over the entire range of viable H-ion concentrations for *Paramecium*. It is also indicated in the table that ciliary reversal is not induced by any of the anions tested, and it is probable that

any action of the anions in ciliary reversal is quantitative, affecting the rate or degree of action of the cations.

DISCUSSION

The experiments summarized here demonstrate that increase in concentration in the environment of monovalent cation salts and hydrates, $BaCl_2$, $Ba(OH)_2$, $MnCl_2$, $MnSO_4$, oxalic acid, and H_2CO_3 induce reversal in the direction of the effective stroke of the cilia in *Paramecium*. Ciliary reversal is not induced by increase in concentration of most bi- and trivalent cation salts and hydrates, mineral and organic acids, and nonelectrolytes. Since the chemical agents doubtless do not create new functions in the cell, it follows that they can only modify existing functions, or at most make evident functions which have previously been latent; and the difference in behavior in the present experiments must be due to some effect on the protoplasm of the organism by the substances inducing ciliary reversal not shared, at least in degree, by the substances which do not.

Unfortunately, there is little direct evidence concerning the effect of chemical agents on the protoplasm of *Paramecium*. However, as has been pointed out by Oliphant (1938) and by Beams and King (1941), experiments of Heilbrunn (1923, 1928, 1937), Heilbrunn and Daugherty (1931), Cholodnyj (1923), Weber (1924), Spek (1921), and others indicate that lithium, potassium, sodium, and ammonium increase protoplasmic viscosity in a number of forms and calcium and magnesium decrease viscosity. The action of other salts has received little attention, and there is no general agreement regarding their effect on protoplasm. Many experiments (see Heilbrunn, 1928, 1937, and Beams and King, 1941, for literature) show that salts of heavy metals are extremely toxic even in very low concentration and that death in solution of salts of heavy metals is accompanied by coagulation of the protoplasm associated with a salting-out of the proteins. Ciliary reversal in salts of heavy metals only occurs just before death, at a time when there can be no question of marked increase in protoplasmic viscosity. Heilbrunn (1928, p. 151) mentions preliminary experiments indicating that $AlCl_3$ and $CeCl_3$ decrease protoplasmic viscosity in eggs of *Arbacia*.

In connection with the present investigation the results of Jacobs (1922) may be illuminating. He observed that short exposure of *Paramecium*, *Colpidium*, and, to a lesser degree, *Spirogyra* and eggs of *Arbacia* to CO_2 is accompanied by a marked decrease in protoplasmic viscosity, as measured by centrifuge tests, while longer exposures markedly increased viscosity. As pointed out above, reversal in ciliary action is induced only by prolonged exposure to CO_2. Jacobs is inclined to attribute the changes to some specific action of CO_2, though he does not say what the action is. Among the nonelectrolytes tested, many investigators (e.g., Heilbrunn, 1937; Beams and King, 1941) have found that fat solvents appreciably decrease protoplasmic viscosity. In the solutions of glucose, sucrose, and urea in which ciliary reversal occurs, there is a marked plasmolysis, which is undoubtedly accompanied by an increase in protoplasmic viscosity.

There is, consequently, considerable evidence which indicates that many, if not all, chemicals which induce reversal in ciliary action also induce an increase in viscosity of the protoplasm of a number of organisms, while most of the chemicals tested which failed to induce ciliary reversal decrease protoplasmic viscosity or fail to measurably affect viscosity. Grave and Schmitt (1925) demonstrated that cilia in mollusks are closely connected with nerve fibers, and microdissection experiments showed that action of the cilia is probably under nervous control. Work of Rees (1922), Lund (1933), Worley

(1934), and others on *Paramecium* suggest that the cilia are somewhat similarly controlled or influenced by a neuromotor apparatus, though the demonstration is by no means so complete. If these statements are true, it is conceivable that an increase in viscosity of the cytoplasm could produce impulses in the neuromotor apparatus resulting in reversal in the action of the cilia. It is also conceivable that, as populations of denervated smooth muscle cells can be induced by chemicals in the environment to violent contractions, so could cilia be induced to reverse the direction of their effective beat in the absence of a neuromotor or similar apparatus.

In addition to viscosity changes, other factors, such as changes in electric potential at the surface or in permeability and consistency of the surface layer of the organisms or chemical changes within the organism, are undoubtedly also involved. How reversal in ciliary action is induced by chemicals cannot be explained on a basis of the present survey or our present knowledge. However, the reversal in direction of the effective beat of cilia is a striking example of contractile behavior of protoplasm which is of widespread occurrence, which is induced by specific agents in the environment, and which can be quantitatively measured. Moreover, this behavior occurs in types of biological material which may be obtained in any desired quantity and whose genetic and environmental factors, recent studies have shown, may be satisfactorily standardized and so critically studied. A further study of ciliary reversal should contribute to the ultimate analysis of the chemicophysical factors involved in the general phenomena of protoplasmic contractility.

SUMMARY

Reversal in the direction of the effective beat of the cilia in *Paramecium* is induced by increase in concentration in the environment of most, if not all, monovalent cation salts and hydrates, $BaCl_2$, $Ba(OH)_2$, $MnCl_2$, $MnSO_4$, oxalic acid, and H_2CO_3. Ciliary reversal is not induced by increase of most bi- and trivalent cation salts and hydrates, mineral and organic acids, and nonelectrolytes.

Ciliary reversal is not directly associated with the osmotic value of the solution inducing it, nor is it directly associated with the hydrogen-ion concentration of the solution.

Ciliary reversal is not induced by any of the anions tested, under conditions of the present experiments; and any action of anions in reversal is probably quantitative, affecting the rate or degree of action of the cations.

Reversal in ciliary action is probably associated with some specific action of the cations in solutions inducing it, and there is some evidence to suggest that increase in protoplasmic viscosity is involved.

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THE EFFECT OF TEMPERATURE ON FOOD-VACUOLE FORMATION IN PARAMECIUM[1]

(Four figures)

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THE effects of temperature on growth, movement, and excretion in the Protozoa have been studied by a number of investigators, but at present the literature contains no data pertaining to the effect of temperature on food-vacuole formation in the genus Paramecium.

The purpose of the present investigation was to devise a method by which the effect of temperature on food-vacuole formation could be studied and to obtain data concerning this effect for *Paramecium caudatum*, *P. aurelia*, and *P. multimicronucleatum*.

MATERIAL AND METHOD

The infusoria which formed the basis of the experimental work were races of *P. aurelia*, *P. caudatum*, and *P. multimicronucleatum*. The latter two species were obtained from the pure-line cultures of Professor R. L. King. The first species was obtained by an isolation from pond water in the vicinity of Iowa City.

The standard culture medium for the three species was prepared as follows: 10 gm. of timothy hay were autoclaved in 1 liter of distilled water under 15 pounds of pressure for 15 minutes.

The lower temperatures (4°, 10°, and 15° C.) were obtained by the use of an electric refrigerator. The variations in temperature were $\pm 1°$ C. The observations on higher temperatures (20° and 40° C.) were made by the use of automatically controlled constant-temperature baths. The variation in the latter was considerably less than 1° C.

The organisms were placed in a balanced salt solution (Gaw, 1935).[2] The pH of this solution was about 6.9.

These organisms lived for 72 hours in this solution without any apparent ill effect. In making the test, 60 cc. of a suspension of the organisms were placed in a 100-cc. bottle in a compartment at different temperatures. Eight of the above tests were made at the same time from the same culture, and these were placed in compartments at 4°, 10°, 20°, 25°, 30°, 35°, and 40° C., respectively. These cultures were usually allowed to remain at the above temperatures for 8 hours in all cases except at 40°, where the time was 2 hours. After the period of acclimatization, India ink was added to the culture, and some of the or-

[1] This paper represents part of a dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Zoölogy of the State University of Iowa, June, 1941.

The writer wishes to express his indebtedness to Professor T. L. Jahn for suggesting the problem and for his interest and help at all times during this investigation. Also, he desires to thank Professor R. L. King for his advice and helpful criticism.

[2] Gaw solution: KNO_3, 1.5 gm.; K_2HPO_4, 0.18 gm.; [$MgSO_4$ $7H_2O$], 0.06 gm.; $FeCl_3$, 0.003 gm.; H_2O, 1,000 cc.

ganisms were fixed after 2, 4, 6, and 8 minutes in Worcester's fluid. The number of food vacuoles per organism which contained the India ink was counted and recorded. An average of about fifty organisms was studied at each time interval employed. This procedure was used for all temperatures studied.

If the vacuoles are formed at a constant rate, one would expect a graph of the number of vacuoles containing India ink to be a straight line when plotted against time. This is true for short time intervals. However, since the ink-containing vacuoles are eventually

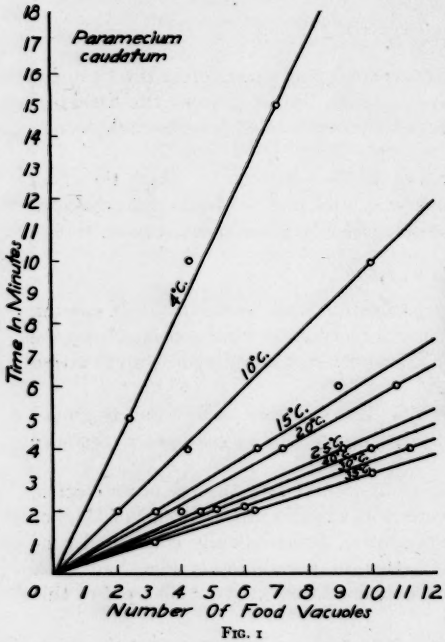

FIG. 1

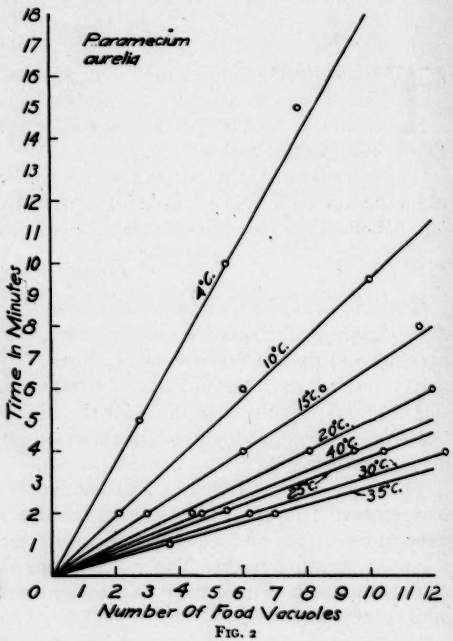

FIG. 2

FIG. 1.—Curve shows a graphic representation of the data of Table 1 for *P. caudatum*, in which the number of food vacuoles is plotted against time at 4°, 10°, 15°, 20°, 25°, 35°, and 40°.

FIG. 2.—Graphic representation of the data of Table 2 for *P. aurelia*

emptied in the anal region, the number of food vacuoles which contain ink will increase linearly only until it approximates the maximum number for the organism. Since food vacuoles are not always eliminated in the same order in which they are formed (Hall and Dunihue, 1930; Dunihue, 1931; and Lee, 1932), and since the number present in an organism may not remain constant, the shape of the curve beyond the linear portion is subject to some variations. However, the linear portion of the curve can be used for calculating the rate of vacuole formation. Calculations based on this procedure have several distinct advantages over observations made on the formation of single vacuoles or on a series of single vacuoles. These are: (1) the organisms are not partly flattened between slide and cover slip, as in the usual microscopical preparation; (2) the organ-

isms are free-swimming under apparently normal conditions; (3) the time necessary for the formation of a large number of vacuoles can be measured and the rate calculated with considerable accuracy.

The rate of food-vacuole formation is equal to the reciprocal of the time necessary to form one vacuole. As a measure of the rate, the time necessary for the formation of ten vacuoles was determined by interpolatiou of the data of Figures 1, 2, and 3. From this was calculated the rate of formation in terms of vacuoles per minute.

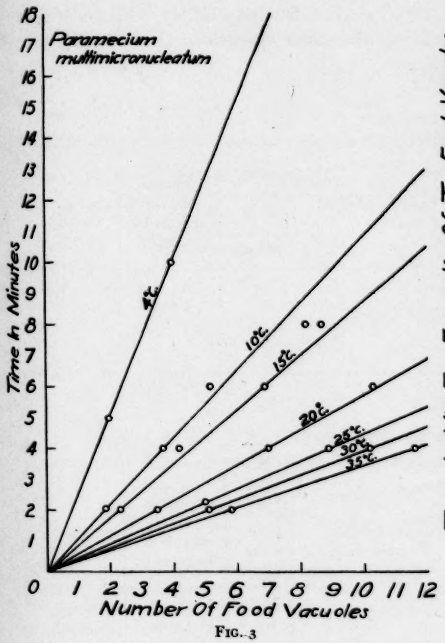

FIG. 3

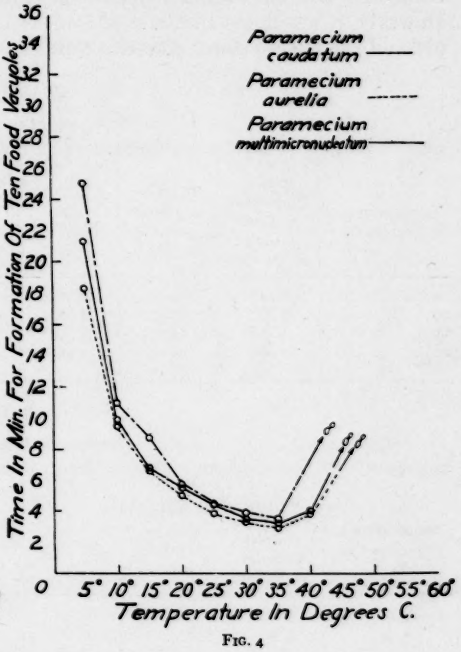

FIG. 4

FIG. 3.—Graphic representation of the data of Table 3 for *P. multimicronucleatum*.

FIG. 4.—Curves show time in minutes for formation of ten food vacuoles plotted against temperature in *P. caudatum*, *P. aurelia*, and *P. multimicronucleatum*.

RESULTS

The results of three typical sets of experiments are shown in Figures 1, 2, and 3 for *P. caudatum*, *P. aurelia*, and *P. multimicronucleatum*. It should be noted that these points closely approximate a series of straight lines. If the duration of the experiment were longer, the curves would bend upward as the older ink-containing vacuoles were eliminated. Each of these points represents the average number of vacuoles in fifty animals.

Three preliminary experiments were performed on the effect of temperature and pH on food-vacuole formation. During these experiments the diameters of food vacuoles were measured in Gaw's solution. It was found that the average diameter of 9,050 food

vacuoles of *P. aurelia* was 9.17 ± 0.33 μ. The average diameter of 6,691 food vacuoles of *P. caudatum* was 14.25 ± 0.10 μ, and that of 13,376 vacuoles in *P. multimicronucleatum* was 17.76 ± 0.20 μ. In another group of experiments the average size of food vacuoles of about one hundred animals of each of the three species was determined at various pH values between pH 5 and pH 9. In a third group of experiments the average size of food vacuoles of one hundred specimens of each species was determined at each of the following temperatures: 4°, 10°, 15°, 20°, 25°, 30°, 35°, and 40° C. It was found that neither the pH nor the temperature had any significant effect on the average size of the vacuoles. However, it was noted that rate of food-vacuole formation varied greatly with both the pH and the temperature. The effect of pH will be discussed elsewhere (Lee, 1942).

TABLE 1

Paramecium caudatum

Temperature in Degrees C.	Rate of Formation in Food Vacuoles per Minute	Temperature Intervals in Degrees C.	Q_{10}	Temperature in Degrees C.	Rate of Formation in Food Vacuoles per Minute	Temperature Intervals in Degrees C.	Q_{10}
4	0.46	4–15	2.87	25	2.27	25–35	1.37
10	1.00	10–20	1.81	30	2.66	35–40	0.65
15	1.47	15–25	1.54	35	3.12	.....	.....
20	1.81	20–30	1.47	40	2.50	.....	.....

TABLE 2

Paramecium aurelia

Temperature in Degrees C.	Rate of Formation in Food Vacuoles per Minute	Temperature Intervals in Degrees C.	Q_{10}	Temperature in Degrees C.	Rate of Formation in Food Vacuoles per Minute	Temperature Intervals in Degrees C.	Q_{10}
4	0.54	4–15	2.54	25	2.59	25–35	1.32
10	1.05	10–20	1.90	30	3.03	35–40	0.60
15	1.51	15–25	1.71	35	3.44	.....	.....
20	2.00	20–30	1.51	40	2.38	.....	.....

The present data demonstrate that *P. caudatum* and *P. aurelia* can ingest food vacuoles between 4° and 40° C. (Tables 1 and 2) and that for *P. multimicronucleatum* the temperature range is between 4° and 35° C. (Table 3). The rate of ingestion increases with increasing temperatures in *P. caudatum* and *P. aurelia* until 35° C. is reached; then it decreases. In *P. multimicronucleatum* an increasing rate of food-vacuole formation was noted up to the maximum of 35°, but 40° C. was lethal.

Tables 1, 2, and 3 show a decreasing Q_{10} with increasing temperature. For food-vacuole formation of *P. caudatum* there is a decrease from 2.82 (between 4° and 15° C.) to 0.65 (between 35° and 40° C.). *Paramecium aurelia* shows a similar decrease from 2.54 (between 4° and 15°) to 0.60 (between 35° and 40°), while *P. multimicronucleatum* shows a decrease from 2.59 (between 4° and 15°) to 1.35 (between 25° and 35°). These data are

summarized in Table 4. Calculations of the temperature coefficients demonstrate a value for frequency of ingestion of food vacuoles of approximately 15,000 calories below 15° and of 7,000–11,000 above 15°.

DISCUSSION

In the present study the effect of temperature on the rate of food-vacuole formation may be considered to be caused directly by the effect of temperature on the action of cilia in the peristome. The μ-values obtained in this study are in close accord with those obtained by Glaser (1924) for forward movement in *Paramecium* sp. However, the present data cannot be used for the purpose of determining the exact location of sharp

TABLE 3

Paramecium multimicronucleatum

Temperature in Degrees C.	Rate of Formation in Food Vacuoles per Minute	Temperature Intervals in Degrees C.	Q_{10}	Temperature in Degrees C.	Rate of Formation in Food Vacuoles per Minute	Temperature Intervals in Degrees C.	Q_{10}
4	0.40	4–15	2.59	25	2.18	25–35	1.35
10	0.90	10–20	1.95	30	2.53	.....	.....
15	1.14	15–25	1.91	35	2.94	.....	.....
20	1.77	20–30	1.42	40	0	.....	.....

TABLE 4

TEMPERATURE COEFFICIENTS AS MEASURED BY RATE OF FOOD-VACUOLE FORMATION

Species	4°–15°		10°–20°		15°–25°	
	Q_{10}	μ	Q_{10}	μ	Q_{10}	μ
Paramecium caudatum	2.87	16,770	1.81	10,162	1.50	6,848
P. aurelia	2.54	14,825	1.90	10,603	1.71	9,080
P. multimicronucleatum	2.59	15,155	1.96	11,137	1.91	11,053

changes in the μ-value because the temperature intervals are 5° C. The present data show a close similarity to the μ-value of 16,500, which Crozier (1924) ascribed to oxidative processes; and it seems quite possible that an oxidative process might control the ciliary activity of the peristome.

Our knowledge of the effect of temperature on ciliary activity in other animals is limited to the data of Umeda (1929)[3] and Gray (1928) on ciliated epithelium. Umeda (1929), studying the effects of temperature on the movement of ciliated epithelium of the ox trachea *in vitro*, found that raising the temperature increased the rate to a maximum at 40° C. and that a temperature of 50° C. destroyed ciliary movement. Gray (1928) made similar observation on *Mytilus*, which showed an increase in ciliary movement as the temperature was raised from 0° to 32.5° C., then a decrease, and finally a

[3] Cited in Rogers, 1938.

complete cessation of ciliary activity at 45° C. He further suggested that a decrease in ciliary activity at higher temperatures may be due to an insufficient amount of oxygen or to a destruction of one or more of the enzymes. Kitching (1939) showed that the rate of movement of *Paramecium* was not decreased by low oxygen tensions until the oxygen pressure became less than 1 mm. Hg at 24°–28° C. Therefore, one might not expect the lack of oxygen to cause a decrease in the case of *Paramecium* at atmospheric pressure and 40° C. The second suggestion of Gray (1928) that the decrease is caused by destruction of endocellular enzymes is still without experimental evidence.

SUMMARY AND CONCLUSION

1. A quantitative method has been devised for studying the effect of temperature on ciliary activity of the peristome.

2. The Q_{10} of members of the aurelia group of *Paramecia* for food-vacuole formation falls between 2 and 3 below 15° C. and between 1 and 2 above 15° C.

3. The temperature characteristics for food-vacuole formation of the aurelia group were about 15,000 calories below 15° C. and from 7,000 to 11,000 calories above it.

4. The formation of food vacuoles, and presumably the ciliary activity of the peristome, of *P. caudatum*, *P. aurelia*, and *P. multimicronucleatum* increases with increasing temperature until 35° C. is reached; then it decreases in rate. A temperature of 40° C. is lethal to *P. multimicronucleatum* but not to *P. aurelia* and *P. caudatum*.

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THE EFFECT OF pH ON FOOD-VACUOLE FORMATION IN PARAMECIUM[1]

(Four figures)

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THE effect of pH on the reproduction of ciliates has been the subject of many investigations, but the effect of pH on food-vacuole formation has been investigated only for *Colpidium* (Mills, 1931). Metalnikow (1912) made some early observations on the effects of acidity and alkalinity on the frequency of food-vacuole formation in *Paramecium* sp., but at that time the methods of measuring hydrogen-ion concentration were quite inadequate.

The feasibility of using the frequency of ingestion of food vacuoles of *Paramecium* as an index of the physiological state of the organism was pointed out by Jacobs (1912), Losina-Losinsky (1931), Bragg (1936), and King and Beams (1941).

The purpose of the present investigation was to study the effect of H-ion concentration on the frequency of ingestion of food vacuoles in *P. aurelia*, *P. caudatum*, and *P. multimicronucleatum*. It is possible that the results of such a study might be useful in interpreting the sequence of Protozoa in hay infusion. The data obtained can be used for comparing the pH ranges and maxima for ingestion with those obtained by other investigators for the growth of each of these species. It is also possible to compare the reactions of three closely related species to identical conditions.

MATERIAL AND METHOD

The members of the aurelia group which form the basis of this experimental work were subcultures of the original stock cultures reported in a previous paper (Lee, 1942). All data were obtained during the growth phase of the cultures.

Buffer solutions of known H-ion concentrations were prepared according to the directions of Clark and Lubs (Clark, 1928). The pH values were checked by means of the colorimetric method.

The buffer used between pH 4.0 and pH 5.5 was potassium acid phthalate and sodium hydroxide. For pH values of 6.0, 6.6. 7.0, and 7.6 the potassium dihydrogen phosphate and sodium hydroxide were used. Above pH 8.0 the buffer used was boric acid, potassium chloride, and sodium hydroxide. Preliminary experiments with potassium acid phthalate at pH 6.0 and the boric acid–potassium chloride–sodium hydroxide mixture at pH 8.0 failed to show any measurable toxic effects. These two buffer mixtures were tested in the nonlethal range in order to ascertain that the lethal effects obtained at pH 4.0 and pH 10.0 were real pH effects and not effects produced by the buffer mixture.

[1] This paper represents part of a dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Zoölogy of the State University of Iowa, June, 1941.

The writer wishes to express his indebtedness to Professor T. L. Jahn for suggesting the problem and for his interest and help at all times during this investigation. Also, he desires to thank Professor R. L. King for his advice and helpful criticism.

Six cubic centimeters of the solution under consideration were added to each of four 12-cc. vials. Several drops of hay infusion containing a thick suspension of *Paramecia* were added to each vial. Ten minutes later India ink was added to each vial. Generally, five drops of the ink were added to each vial, and care was taken to mix the content of the vials to assure an even distribution of the ink particles. The organisms in the four vials were fixed at the end of 1, 2, 4, and 6 minutes by the addition of Worcester's fluid or a modified Noland's fluid. The latter fixative consists of 85 cc. of saturated phenol

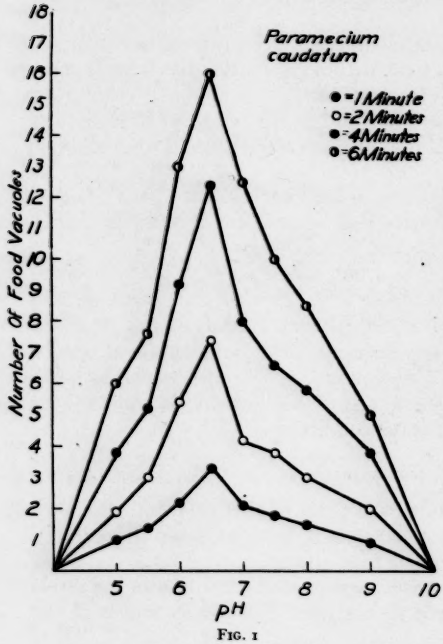

FIG. 1

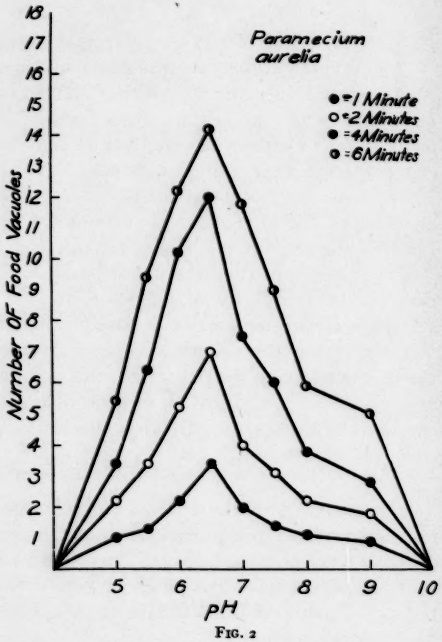

FIG. 2

FIG. 1.—Curve shows a graphic representation of the data of Table 1 for *P. caudatum*, in which the various H-ion concentrations were plotted against the number of food vacuoles formed in 1, 2, 4, and 6 minutes, respectively.

FIG. 2.—Graphic representation of data of Table 2 for *P. aurelia*.

and 15 cc. of commercial formalin. The contents of each vial were examined, usually under the low power of the microscope, and the number of food vacuoles containing India ink in each organism was determined. The average of fifty organisms was determined at each time interval. All experiments were performed between 20° and 22° C.

RESULTS

Preliminary experiments were performed which showed clearly that pH affected the rate of food-vacuole formation and not the diameter of the vacuoles. The rate was ascertained by plotting the number of food vacuoles formed against time at various H-ion

concentrations. The details and advantages of this method have been explained in a previous paper (Lee, 1942). Figure 4 is a graph showing the above relationship between pH and the rate of food-vacuole formation for *P. caudatum*.

Figure 1 shows an optimum for the frequency of ingestion of food vacuoles at pH 6.6 for *P. caudatum*. No vacuoles were formed at pH 4.0 or pH 10.0. The curve for *P. aurelia* (Fig. 2) is very similar, with the optimum also at pH 6.6. However, the optimum for *P. multimicronucleatum* was at pH 7.0 (Fig. 3).

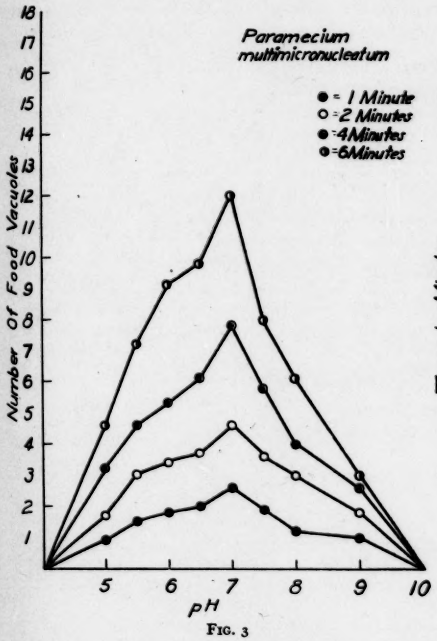

FIG. 3

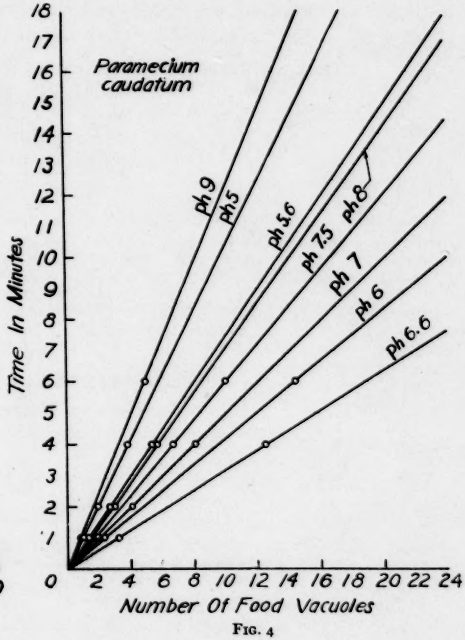

FIG. 4

FIG. 3.—Graphic representation of data of Table 3 for *P. multimicronucleatum*.

FIG. 4.—Curves showing the number of food vacuoles plotted against time in *P. caudatum* at the following pH values: 4.0, 5.0, 5.6, 6.0, 6.6, 7.0, 7.6, 8.0, 9.0, and 10.0. The rate of food-vacuole formation was interpolated for the above pH values. The rate of food-vacuole formation in *P. aurelia* and *P. multimicronucleatum* was calculated in a similar manner.

Members of the aurelia group in general show a close similarity in the effect of pH on the frequency of ingestion of food vacuoles. *Paramecium caudatum* has the highest optimum of sixteen food vacuoles in 6 minutes; *P. aurelia* is next, with fourteen vacuoles in the same length of time; and *P. multimicronucleatum* produces only twelve food vacuoles in 6 minutes. This might be taken to indicate that there is a difference in ciliary activity of the peristome in the present species of *Paramecium*. Table 1 shows how the time required to form one food vacuole varies with the H-ion concentration. These values were computed from data of Figures 1, 2, and 3.

No food vacuoles were formed at pH 4.0 or at pH 10.0. During the course of observations on organisms at these pH values it was noted that all the organisms died within a few minutes and that *P. multimicronucleatum* seemed to be more resistant. This difference in resistance was checked by determining the survival time of different species. The results are shown in Table 2. The survival time was taken as the time necessary for twenty-five out of fifty organisms to become motionless. It can be seen from the table that the order of resistance to the lethal pH values of both 4.0 and 10.0 was: *P. multimicronucleatum* > *P. caudatum* > *P. aurelia.*

TABLE 1

TIME (IN MINUTES) NECESSARY FOR FORMATION OF ONE FOOD VACUOLE AT VARIOUS H-ION CONCENTRATIONS

SPECIES	pH							
	5.0	5.6	6.0	6.6	7.0	7.5	8.0	9.0
Paramecium caudatum	1.01	0.72	0.40	0.31	0.52	0.63	0.70	1.20
P. aurelia	1.10	0.74	0.41	0.33	0.54	0.68	0.78	1.31
P. multimicronucleatum	1.20	0.84	0.60	0.54	0.50	0.58	0.80	1.15

TABLE 2

EFFECTS OF LETHAL pH ON SURVIVAL TIME

(Survival Time in Minutes)

SPECIES	pH	
	4	10
Paramecium aurelia	0.80	2.10
P. caudatum	0.99	3.10
P. multimicronucleatum	2.00	5.40

DISCUSSION

The literature on the effect of pH on ciliary activity is apparently limited to several papers. Gray (1928) studied the effect of pH on activity of the cilia of the gills of *Mytilus* when the gills were immersed in sea water. He found that the cilia were inactive below pH 5 and above pH 9.5. Chase and Glaser (1930) studied the rate of forward movement of *Paramecium* sp. as a function of pH with various acids and alkalies. With valeric and carbonic acids they found an optimum at pH 5.5–6.0, with a lower rate at pH 5.0 and a much lower rate at pH 8.0. With hydrochloric and sulphuric acids they found that the effect of pH was temporary and that after 3 or 4 hours the rate was the same at all pH values between 5.4 and 7.0. They explained these effects on the basis of an ampholyte, the concentration of which determined the rate of ciliary activity and thereby the rate of forward movement. In the present data a definite effect was observed with phosphate buffers, which one might expect to act like hydrochloric or sul-

phuric acid rather than like valeric or carbonic acid. However, the present experiments were performed over a wider pH range than Chase and Glaser's experiments. The present experiments could be explained on the basis of a superficial amphoteric electrolyte, as suggested by Chase and Glaser; but at present there seems to be no evidence that such is the case. Mills (1931) maintained that in *Colpidium* sp. the optimum H-ion concentration for food-vacuole formation is pH 6 (Table 3). She observed the formation of mucus in *Colpidium* and explained that the low number of food vacuoles at certain H-ion concentrations may be due to the retardation of the cilia by the mucus. Present data of the author show that mucus was secreted in members of the *P. aurelia* group at sublethal H-ion concentrations and that Mills's (1931) explanation might be applied to the present species of *Paramecium*.

TABLE 3

KNOWN EFFECTS OF pH ON FOOD-VACUOLE FORMATION AND GROWTH IN CILIATES

Species	Non-Bacteria-Free Growth		Bacteria-Free Growth		Food Vacuoles	
	pH	Author	pH	Author	pH	Author
Colpidium sp.	.....	.....	6.7	Elliott,* 1933	6.0	Mills, 1931
Colpoda cucullus	.....	Morea, 1927	.....	.....	.....	.....
Paramecium aurelia	7.0	Morea, 1927	.....	.....	6.6	Present results
	6.7	Darby, 1929	.....	.....	.....	.....
	None	Phelps,† 1931	.....	.....	.....	.....
P. caudatum	7.0	.....	.....	.....	6.6	Present results
P. caudatum	6.0–8.0	Losina-Losinsky, 1931	.....	.....	.....	.....
P. multimicronucleatum	7.0	Jones, 1932	.....	.....	7.0	Present results
P. bursaria	.....	.....	6.7	Loefer, 1938	.....	.....
Stylonychia pustulata	6.7	Darby, 1929	.....	.....	.....	.....
Glaucoma ficaria	.....	.....	6.5	Johnson, 1935	.....	.....
G. piriformis	.....	.....	4.8	Johnson, 1935	.....	.....

* *Colpidium striatum.*

† *Paramecium aurelia* showed same division rate between 5.9 and 8.2.

It seems possible that there should be a close correlation between the rate of food-vacuole formation and the growth of *Paramecium* (Table 6). Whenever India ink is added to a culture of *Paramecium*, it is found that almost every individual contains black food vacuoles within a very few minutes. This indicates that food vacuoles are being formed in all organisms almost continuously. It is possible that the rate of food-vacuole formation may, at least under some circumstances, control the rate of growth.

Since pH is one of the factors which control the rate of food-vacuole formation, it seems possible that one effect of pH on growth might be an indirect one through the mechanism of food-vacuole formation. If this hypothesis were true, we should expect the pH for maximum rate of food-vacuole formation to be the same as that for growth. The known effects of pH on food-vacuole formation and growth in ciliates is shown in Table 3. It can be seen from the table that there is a close correlation between the optima for food-vacuole formation and for growth. However, since in all cases where both data are available the optimum is close to neutrality, this correlation may be spurious. It would

be interesting to investigate the effect of pH on food-vacuole formation in *Glaucoma piriformis* because in this species the optimum for growth is at pH 4.8.

Woodruff (1912) suggested that the sequence of Protozoa in hay-infusion cultures may be due to a shift in the kind of available food; Bodine (1921) suggested some correlation between the H-ion concentration and the sequence of Protozoa in hay infusion. The results of the writer seem to show that pH produces a direct effect on the mechanism of food-vacuole formation. It is possible that the sequence of protozoans in hay infusion might be caused by the pH altering the intake of food and thereby gradually producing a condition of starvation. As the H-ion concentrations are removed from the optimum, a decrease in food-vacuole formation becomes obvious (Figs. 1, 2, 3). This might be responsible for the decrease in division rate noted by Darby (1929) in hay infusion.

Physiological differences between species of *Paramecium* have been reported by other workers. Unger (1926) and Gaw (1936) demonstrated a difference in the action of contractile vacuole in various species of *Paramecium*. Geise and Reed (1940) have shown a difference in susceptibility of the aurelia group to ultra-violet radiation. In a previous paper (Lee, 1942) it has been pointed out that there are differences in the effect of temperature on food-vacuole formation, and in the present paper it is demonstrated that there is a difference in the effect of pH on the rate of food-vacuole formation and in the resistance of the organisms to solutions of lethal pH. It seems possible that there may be other, as yet undetermined, differences between these species. Very definite physiological differences have been reported between the various species of *Euglena* (review Jahn, 1934) and of *Strigomonas* (review, Jahn, 1941) and perhaps other genera. Hall (1941), in his extensive review of the literature, has cited numerous examples of physiological differences in species according to their nutritional requirements. It seems possible that in some cases physiological differences may become useful as a method of differentiating various species.

SUMMARY AND CONCLUSION

1. A quantitative method was used for studying the relationship between food-vacuole formation of *Paramecia* and pH.

2. It was found that the optimum pH for rate of food-vacuole formation in *P. caudatum* and *P. aurelia* was 6.5 and for *P. multimicronucleatum* was 7.0. No food vacuoles were formed at pH 4.0 or pH 10.0 by any of these species.

3. The susceptibility of the three species to lethal pH values (pH 4 or pH 10) was determined. *Paramecium multimicronucleatum* was the most resistant, *P. aurelia* the least resistant, and *P. caudatum* was intermediate.

4. *Paramecium caudatum* showed the highest rate of food-vacuole formation at all pH values studied, while *P. aurelia* had the next highest rate, and *P. multimicronucleatum* the lowest rate.

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AN INVESTIGATION OF THE PHYSIOLOGICAL REQUIREMENTS OF A PURE CULTURE OF THE HETEROTROPHIC FLAGELLATE, OIKOMONAS TERMO, KENT[1]

(One figure)

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DURING the course of some ecological investigations it became desirable to obtain a pure culture of the common soil and fresh-water flagellate *Oikomonas termo*, Kent. The pure culture was obtained and a rough survey made of the cultural conditions required for its growth. The results of this survey have suggested a number of interesting physiological questions which will be pointed out in the course of this report.

A METHOD FOR OBTAINING A PURE CULTURE OF THE FLAGELLATE

Although it probably occurs in virtually all soils and ponds, *O. termo* is often difficult to find in a sample which is examined directly. To obtain it in considerable quantities one must use an enrichment culture. Almost any infusion commonly used in protozoölogical work will do. Of a number tried I have found a 1 per cent rolled-oat medium the best. This medium can be prepared as follows: 2 gm. of Quaker Oats are added to 100 ml. of distilled water, which is then heated to 70° C. and held at that temperature for a few minutes. When it is cool, the supernatant fluid is diluted with an equal quantity of the water sample which is to be tested for the presence of *Oikomonas*. The depth of the medium should not be over 2 cm. If *Oikomonas* is present, it will multiply tremendously during the first few days and will collect in greatest concentration in the surface film.

The flagellate can be obtained in pure culture fairly easily by the washing technique developed by Hargitt and Fray (1917) for *Paramecium*. Because *Oikomonas* is quite small (5–20 μ in greatest dimension) one must modify the usual procedure. By using a dissecting binocular microscope of moderate power (a linear magnification of 20× is sufficient) and by balancing the light coming from the mirror below against that which enters the culture medium from the side, one can make the monads visible—or, rather, one can see tiny points of light which slowly move in circles. With sterile capillary pipettes such a point of light can be transferred from dish to dish of sterile medium until the animal is free of other organisms. Using watch glasses which held about 1 ml. of fluid and very fine capillary pipettes I found five washes sufficient to sterilize six out of seven animals so washed. Only one of the resulting pure cultures was used in the subsequent experiments.

The purity of this culture was checked with a large variety of media involving yeast autolysate, yeast extract, yeast water, beef extract, peptone, blood, blood serum, gela-

[1] For much help and criticism I am indebted to Dr. W. H. Johnson, under whose direction this research was carried out. I also wish to thank Dr. C. B. van Niel for his advice and criticism.

[2] Now at Carnegie Institution of Washington, Division of Plant Biology, Stanford University, California.

tin, liver infusion, and potato infusion. Many of these media were also made with an added carbohydrate, such as dextrose, lactose, sucrose, maltose, and glycerol. Various concentrations of nutrients were used; and in every case except that of the blood medium, which was prepared only as an agar, both a broth and an agar medium were used. These tests were all carried out aerobically. In addition somewhat less extensive anaerobic tests were carried out using only broths. All tests were set up in triplicate and microscopically examined for a month. No contaminants appeared, other than sporadic molds on the agar plates. In addition the diverse media employed in the following experiments and the nutrient agar test plates made of some of the cultures of every experiment constitute further sterility tests, which also failed to reveal any contaminating organisms. Therefore, I consider it reasonably certain that the experiments reported here were carried out with a pure culture.

DETAILS OF THE EXPERIMENTAL PROCEDURE

Bacteriological sterile techniques were used throughout. Media were sterilized at 14 lb. pressure for 20–25 minutes. Inoculations were made with platinum or platinum-iridium loops or with sterile pipettes. Soft-glass tubes were ordinarily used, though sometimes Pyrex was employed. In parallel experiments run with the two types of glass no difference in growth was detectable through four transfers. However, in the experiments which follow, all the tubes in any one experiment were of the same kind of glass.

Chemicals were of the "C.P." or "Reagent" quality. Triple (glass) distilled water was used throughout, though single distilled water seemed to be just as good with the organic media used. The depth of the medium in a tube was generally about 4 cm. The pH was determined colorimetrically or with a Beckman pH meter, for the use of which I am indebted to Mr. William E. Berg and Dr. D. M. Whitaker. For colorimetric determinations, only one decimal place is given, e.g., pH 6.2. If the Beckman instrument was used, two decimal places are indicated, e.g., pH 6.21.

The temperature varied from experiment to experiment. Thermostatically controlled incubators, with a variation of $\pm 1°$ C. were used much of the time. Of the few temperatures tried (namely, 25°, 30°, 37°, and 40° C.) 30° C. was found to be best. Whenever possible, experiments were run at that temperature; but intermittent trouble with the thermostats made it necessary to run many of the experiments at room temperature, which varied between 17° and 25° C.

Growth of the monads was estimated by simple visual inspection. The tubes were held against a strong light and the turbidity estimated. To record the amount of growth, +'s were used, the number of +'s being indicative of the relative turbidity of the culture. If growth was slight or doubtful, it was recorded thus: (+). This is only a roughly quantitative system of notation: +++ is greater than ++, but it must not be inferred that it is 50 per cent greater. Furthermore, +++ in one experiment cannot be considered equal to +++ in another. Comparisons are to be made only *within* an experiment.

The assumption that the turbidity is a measure of the density of the population is not without fault. Oikomonads of different size (see Fig. 1 as evidence of the variability of this species) and internal consistency will undoubtedly affect the transmission and refraction of light differently. Also, their tendency to clump varies with the cultural conditions, thus increasing the subjective errors. For exact work, accurate counts of organisms should be made. But for a first survey I believe that estimation of growth by turbidity is sufficiently accurate, in spite of its undeniable shortcomings.

THE CALCIUM AND MAGNESIUM REQUIREMENTS OF *Oikomonas*

Oikomonas termo was first isolated in very dilute (0.2 per cent) yeast autolysate, but it was soon found that a 0.5 per cent solution of Difco proteose peptone gave better growth. Random tests of several substances revealed that the addition of glycerol (1 per cent) improved the growth. However, even with this medium, growth was never very good. The road to the finding of a completely satisfactory medium was not opened until the more or less accidental discovery that the addition of a slight amount of agar—as

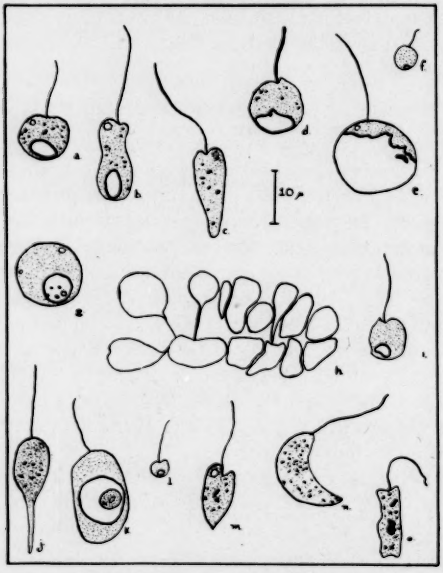

FIG. 1.—Illustrating the variability of *Oikomonas termo*, partly caused by different cultural conditions. All the animals illustrated are from the same clone. The "typical" forms are shown in *a* and *b*. Note the anterior contractile vacuole. The hyaline body in the posterior part is probably a reserve body. The various shapes that may be assumed by the members of a clump of flagellates are seen in *h*. In *j* may be seen the temporary attachment pseudopod or pedicle. The extremes of size are shown in *k* and *l*.

little as 0.03 per cent—markedly improved the growth. Similar improvement in growth could be achieved by the addition of a water extract of agar or of a small amount (5 parts per hundred) of sterile sea water.

Since the unmodified peptone-glycerol medium contained no salts other than those introduced with the proteose peptone, it seemed not unreasonable that the beneficial effect of agar or agar extract or sea water might be attributable to the presence of inorganic ions. It has been known for some time (see McIlwain, 1938) that agar is an organic salt of calcium and magnesium. The metals can be released only by the hydrolysis of the agar. Since a certain amount of hydrolysis results from the usual autoclaving of agar, some calcium and magnesium may be made available in an agar-containing

medium. The following experiment was based on the inference that it was these ions which accounted for the beneficial effect of agar.

The effect of the addition of various amounts of $CaCl_2$ and $MgCl_2$ to a glycerol-peptone medium was tested. The tubes of media were inoculated and incubated at 30°, and readings were made after 5 days' growth. From the results recorded in Table 1 it is evident that, when no magnesium was added, even as little as 0.005 per cent $CaCl_2$ noticeably improved growth. With $MgCl_2$ added, there was less need for the addition of calcium. There is no evidence in these results of an antagonistic effect of these two salts.

When no magnesium is added, it is remarkable how much calcium is needed to give really excellent growth—0.02 per cent $CaCl_2$, or approximately 0.0018 mole. This is more than ten times as much $CaCl_2$ as Peters' medium for protozoa contains, and more than one hundred times as much as there is in Osterhout's salt solution. Of course, the $CaCl_2$ apparently required by *Oikomonas* in the case just considered may be as high as it is partly because no other salts were added. But it is noteworthy that other experi-

TABLE 1

COMBINED EFFECT OF CALCIUM AND MAGNESIUM ON THE GROWTH OF *Oikomonas*

Percentage of $MgCl_2$	Percentage of $CaCl_2$					
	0	0.005	0.01	0.02	0.04	0.08
0	−	+	++	+++	+++	++++
0.04	(+)	+++	++++	++++	++++	+++++
0.08	−	+++	++++	++++	+++++	++++
0.16	−	++	+++	++++	++++	+++

ments showed Osterhout's solution to be a poor medium unless a comparable quantity of $CaCl_2$ was added.

It is interesting to compare the calcium requirement of *Oikomonas* with that of two other flagellates (both of which contain chlorophyll) which have been reported to require similarly great concentrations of calcium. Dusi (1933), studying five species of *Euglena* —namely, *E. stellata*, *klebsii*, *anabaena*, *deses*, and *pisciformis*—found that *E. stellata*, but none of the others, required calcium in about the concentration reported here for *Oikomonas*. The medium Dusi used was a mineral solution plus ammonium phosphate. When the phosphate was replaced by peptone, the calcium need was no longer evident.

A similar need for calcium was reported in the case of *Hyalogonium klebsii* by Pringsheim (1937). In his experiments the medium contained sodium acetate (0.2 per cent) and Bacto Tryptone (0.1 per cent). If 0.1 per cent yeast extract was added, the need for calcium was not quite so pronounced.

For easy comparison the relative growth of the three flagellates in various concentration of $CaCl_2$ have been brought together in Table 2. Though different symbols have been used in the three cases, it is apparent that the calcium need is much the same for all three organisms, with *Oikomonas* showing the greatest tolerance for high concentrations of calcium chloride. The minus sign entered for *Oikomonas* growth in the medium containing no added calcium may be misleading. This rating was based on a reading

made after 5 days' incubation of the culture. With a longer period of incubation some growth occurs even in this medium. That is not to say that calcium is not required by this monad, however, for proteose peptone, being a meat digest, undoubtedly contains appreciable quantities of calcium. For a refined deficiency experiment a completely synthetic medium is needed.

TABLE 2

THE EFFECT OF CALCIUM ON THE GROWTH OF THREE FLAGELLATES

Percentage of $CaCl_2$	*Euglena stellata**	*Hyalogonium klebsii*†	*Oikomonas termo*‡	Percentage of $CaCl_2$	*Euglena stellata**	*Hyalogonium klebsii*†	*Oikomonas termo*‡
0	very poor	−	−	0.03		+	
0.001	very poor	±		0.04			+++
0.002	poor			0.05	poor		
0.005			+	0.08			++++
0.01	good	+	++	0.1	very poor	±	
0.02	good		+++				

* Dusi (1933). Medium: mineral solution plus $(NH_4)_3PO_4$.

† Pringsheim (1937). Medium: acetate-tryptone. ± means poor growth.

‡ Personal data. Medium: glycerol-peptone.

FURTHER SALT REQUIREMENTS

The ideal investigation of the salt needs of an organism would be one in which all the salts were handled as variables at the same time. In this way there would be the least chance of overlooking additive, substitutive, or antagonistic effects. But such experimentation would require too much time and equipment to be practicable. Two variables are generally the most that can be conveniently handled.

TABLE 3

COMBINED EFFECT OF SODIUM AND POTASSIUM ON THE GROWTH OF *Oikomonas*

Percentage of KCl	Percentage of NaCl					
	0	0.05	0.1	0.2	0.4	0.8
0	+	+	++	++	++	−
0.01	++	+++	++++	+++	+++	−
0.02	+++	++++	++++	++++	+++	−
0.04	+	+++	++++	+++	++	−
0.08	+++	+++	+++	++	++	−

In the following experiments the calcium and magnesium needs were *ex gratia* taken to be independent of the needs for other ions, specifically, of the sodium and potassium ions. The basal medium was glycerol (1 per cent), proteose peptone (0.5 per cent), $CaCl_2$ (0.01 per cent), and $MgCl_2$ (0.01 per cent). To this various amounts of NaCl and KCl were added. After sterilization the media were inoculated with *Oikomonas* and incubated at 30° for 6 days. The amount of growth then visible is recorded in Table 3. It is

clear that both sodium and potassium improve the quality of the medium, with the optimum concentration of NaCl lying between 0.05 and 0.1 per cent, and for KCl between 0.01 and 0.02 per cent.

With this experiment the systematic investigation of the salt requirements of *Oikomonas* was discontinued. Only one other simple experiment was carried out, a comparison between Medium A, a medium indicated as being the best by the preceding experiments, and Medium No. 5, which is simply Medium A slightly modified to include sulphate and phosphate ions. To both these media were added 1 per cent glycerol and 0.5 per cent proteose peptone. Medium No. 5 proved to be slightly the better of the two; consequently, it was used for all further experiments.

	Percentage of Medium A	Percentage of Medium No. 5		Percentage of Medium A	Percentage of Medium No. 5
KCl	0.01	0.01	$MgSO_4$		0.01
NaCl	0.05	0.05	K_2HPO_4		0.004
$CaCl_2$	0.02	0.02	NaH_2PO_4		0.006
$MgCl_2$	0.02	0.01			

CARBON SOURCES FOR *Oikomonas*

It has been mentioned that *Oikomonas* does not grow well on peptone alone but needs an additional carbon source. Once the salt medium was standardized, it became desirable to determine the suitability of a number of different auxiliary carbon sources.

The basal medium used consisted of 0.25 per cent proteose peptone in Medium No. 5, to which was added 1 per cent of the substance being tested. Pyrex tubes were used, and the basal medium and the solutions of the carbon sources were autoclaved separately, then mixed, after autoclaving to give the correct proportions. The pH of the media was approximately 6.5. The growth achieved during 7 days' incubation at 30° is recorded in Table 4. Dextrose is evidently the best of the foods tried.

TABLE 4

SUITABILITY OF VARIOUS CARBON SOURCES FOR THE GROWTH OF *Oikomonas*

Added Carbon Source	Growth (1 Week)	Added Carbon Source	Growth (1 Week)
Control: no addition	(+)	Lactose	++
Dextrose	+++++	Glycerol	++
Sucrose	+++	Calcium malate	+

NITROGEN SOURCES FOR *Oikomonas*

In assaying the suitability of various substances as nitrogen sources, the basal mineral medium was altered by the addition of "mineralized sea water." The reason for this modification follows.

Where a complex, ill-defined medium (such as a peptone medium) is used, there are present in the medium not only energy sources but also unknown salts. If the peptone is replaced by a simple nitrogen source (such as chemically pure $NaNO_3$, for instance),

not only is a complex nitrogen source removed but so are its contaminating salts. Growth may fail to take place with a quite suitable nitrogen source, simply because of the lack of certain needed salts. In the absence of adequate information about the complete mineral needs of an organism in the presence of any usable energy source, it is best, perhaps, to use what might be called a "shotgun dose," putting into the medium a mixture of all the ions known to be important, as well as others which may be. Soil extract, ignited soil, and pond water are sometimes used for this purpose. A better, more reproducible mixture seems to me to be "mineralized sea water," that is, sea water which has been stored in a dark or poorly lighted place for several months in order that its energy sources may be reduced to a minimum by microbial digestion. Such sea water was used in the following experiment.

The basal medium was salt Medium No. 5 plus 1 per cent mineralized sea water, 1 per cent dextrose, and a nitrogen source in the concentration stated in Table 5. The pH of

TABLE 5

SUITABILITY OF VARIOUS NITROGEN SOURCES FOR THE GROWTH OF *Oikomonas*

Per-centage	Nitrogen Source	Growth (1 Week)	Per-centage	Nitrogen Source	Growth (1 Week)
0.2	Control: no addition	+	0.2	Aspartic acid	+
	Bacto Neopeptone	+++++		Cystine	++
	Bacto Proteose Peptone	+++++		$NaNO_2$	−
	Bacto Tryptone	++++		$NaNO_3$	−
	Bacto Peptone	+++		NH_4Cl	−
	Bacto Yeast Extract	++++		NH_4NO_3	−
	Loeffler's blood serum	++			
	Acid hydrolyzed gelatin	++	0.02		
	Asparagin	++		Asparagin	++
	Tryptophane	+		Tryptophane	++
	Leucine	++		Leucine	++

the medium before addition of the nitrogen source was 5.8. The buffering effect of many of the nitrogenous substances used undoubtedly brought the pH close to 7.0. As will appear in a subsequent section, both of these hydrogen-ion concentrations are within the range suitable to the growth of *Oikomonas*.

The cultures were kept at room temperature. The relative amounts of growth apparent on the seventh day are noted in Table 5.

It is evident that good growth was realized only with the peptones, Difco Neopeptone being best and Difco Proteose Peptone a close second. Growth with the amino acids used was very slight; it was not apparent macroscopically; but in some cases it was clearly greater than in the control culture, as determined by microscopic examination, and in such cases a ++ rating was given. The inorganic nitrogen sources appeared to be toxic in the concentrations used in this experiment.

If the slight growth of *Oikomonas* in the amino acid cultures is ignored, then this organism falls into Lwoff's (1932) class of *haplométatrophes* or into Hall's (1939) "heterometatrophs," consisting of organisms which require an organic carbon source and which cannot grow at the expense of any nitrogenous substance simpler than peptones. It may be that more experimentation will show that, given the proper growth factors or vita-

mins, *Oikomonas* can utilize a nitrogen source simpler than peptone. The slight growth in the amino acids is suggestive in that it points to the utilization of these substances by the monad. But until a synthetic diet is discovered for this flagellate, *O. termo* must be considered a heterometatroph.

THE GROWTH OF *Oikomonas* AT DIFFERENT HYDROGEN-ION CONCENTRATIONS

In investigating the range of hydrogen-ion concentrations over which *Oikomonas* can grow, the nutrient medium was simplified somewhat by the elimination of the less important salts. Only $CaCl_2$ and $MgCl_2$ (0.02 per cent of each) were added to a medium containing glycerol[3] (1 per cent) and proteose peptone (0.5 per cent). This medium was distributed among a number of Pyrex flasks and sterilized. The different hydrogen-ion concentrations were obtained by adding to the various flasks different quantities of HCl or NaOH which had been sterilized separately in Pyrex test tubes. After this adjustment

TABLE 6

GROWTH OF *Oikomonas* AT DIFFERENT HYDROGEN-ION CONCENTRATIONS

A Initial pH of Control	B Final pH of Control (1 Week)	C pH of Exper. after 1 Week	D Change in pH (C−B)	E Growth (2 Weeks)
4.00	3.98	4.08	+0.10	+
4.50	4.46	4.69	+0.23	+++
6.02	5.98	6.24	+0.26	++
6.20	6.12	6.27	+0.15	++
6.46	6.32	6.61	+0.29	+++
6.93	6.79 (2 weeks)	6.86 (2 weeks)	+0.07 (2 weeks)	++ (2 weeks)
7.53	7.82	7.79	−0.03	(+)
8.24	8.20	8.21	+0.01	(+)
9.31	8.88	8.74	−0.14	(−)

the media were then tubed. The experiment was not begun until several days had been allowed for equilibration of the media with the CO_2 of the air. Then one tube of each group was used for a pH determination, one was kept sterile (control tube), and the other two were inoculated with *Oikomonas*. After a period of time (as indicated in Table 6) the pH of one of the experimental tubes was noted and compared with the control. At the same time the amount of growth in the experimental tube was estimated.

It will be noted in the table that although *O. termo* grew well when the glycerol-peptone medium was acid or near neutrality, it grew very poorly in the alkaline media—so poorly that 2 weeks were allowed before the alkaline cultures were considered worth examining. It is also evident that the acid media were always made more alkaline, that is, less suitable for the monad, as a result of the growth of *Oikomonas*. The changes in the alkaline media are probably not significant, since the growth of these cultures was so slight.

The fact that these experimental data imply that *Oikomonas* flourishes only in neutral

[3] At the time this experiment was performed it was not yet known that dextrose was a better carbon source than glycerol; hence the use of glycerol.

or acid environments raises an interesting problem: How does it happen that *Oikomonas* can be found (by the employment of an enrichment culture) in almost any pond water, even when the pH of the water is around 8.0? Does this mean that there may be some medium other than glycerol-peptone which will support the growth of the flagellate in alkaline solution? Or that *Oikomonas* is indifferent to pH when feeding on bacteria? Or that even in nature the monad is restricted to microenvironments which are acid because of bacterial activities? Further ecological and physiological research is needed to answer these questions.

THE RELATIVE INDIFFERENCE OF *Oikomonas* TO VARYING OSMOTIC PRESSURE

The ability of *Oikomonas* to grow in different concentrations of sea water was investigated. The different concentrations were secured by mixing various amounts of two nutrient solutions, one of which had been made with distilled water and sterilized in the autoclave, the other having been made with "mineralized sea water" and sterilized with a Chamberland filter. Both media contained 1 per cent each of glycerol and peptone. Because of the pronounced buffer action of the peptone the difference in hydrogen-ion concentration of the two solutions was negligible, the pH of the distilled-water medium being 6.92, that of the sea-water medium being 6.97.

Media of different concentrations of sea water were prepared and their sterility assured before inoculation. Then each medium was inoculated with one loopful of a stock culture of *Oikomonas* and the cultures kept at room temperature. Under these conditions *Oikomonas* survived a direct transfer into, and grew in, 45 per cent sea water. Attempts to get it to grow in concentrations of 50 per cent or greater were unsuccessful, even when the 45 per cent culture was used as the source of the inoculations.

From these results one might infer that *Oikomonas* is less tolerant of high osmotic pressures than are the Protomonadina investigated by Finley (1930). He found that *Bodo uncinatus* could withstand direct transfer to 100 per cent sea water and that two species of *Monas* could be directly transferred to 50 per cent sea water and gradually acclimated to 100 per cent. However, Finley's work was with bacterially contaminated cultures, whereas my study was made with a bacteria-free culture. Is this an important difference? If bacteria are present, will the effect of osmotic pressure on the monads be different?

With this question in mind, the experiment was repeated, this time using sea water which had not been sterilized. Because the monad could not compete with bacteria in the concentrated glycerol-peptone medium, the glycerol concentration was reduced to 0.2 per cent and that of the peptone to 0.1 per cent. In this medium bacterially contaminated cultures of *Oikomonas* withstood an immediate transfer into 60 per cent sea water and could be acclimated to 100 per cent. A little calculation shows that this difference in behavior of the monad cannot be accounted for by the difference in osmotic pressure of the concentrated nutrient medium used in the pure-culture experiment and the dilute nutrient medium used with the impure cultures. The work of Doudoroff (1940), showing that for the bacterium *Escherichia coli* an increase in the concentration of the nitrogenous food increases the toxicity of salts, suggests that perhaps pure cultures of *Oikomonas* might grow in 100 per cent sea water if the nutrients were more dilute. Unfortunately, a medium containing only 0.2 per cent glycerol and 0.1 per cent peptone is too dilute for appreciable growth of *O. termo*. Some other mode of approach to the problem is needed.

SUMMARY

1. *Oikomonas termo* has been obtained in pure culture.

2. This flagellate grows well in a mineral medium which contains peptone and a carbohydrate such as dextrose.

3. In glycerol-peptone medium it requires a considerable quantity of calcium—nearly 0.002 molar solution of $CaCl_2$—if good growth is to be achieved.

4. *Oikomonas* grows well in glycerol-peptone medium only if the medium is neutral or acid.

5. The flagellate will grow when transferred directly to a glycerol-peptone medium in 45 per cent sea water but not in higher concentrations. However, cultures contaminated with bacteria and growing in dilute glycerol-peptone will grow in 100 per cent sea water.

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THE REPRODUCTION OF TRIBOLIUM CONFUSUM DUVAL IN A SEMISYNTHETIC WOOD-DUST MEDIUM[1,2]

(Two figures)

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THE general problem of nutritive requirements of living organisms is a highly complex one, however it may be approached. It is obvious to the biologist that the effects of this aspect of the environment may be adequately understood only in its relation to the entire economy of the organism and that the criteria made use of in any attempt to assay the suitability or inadequacy of a given diet is a matter whose importance can hardly be overestimated. Studies on mammalian nutrition have, for the most part, been made in terms of the growth rate, as measured by weight gains, of the immature animal, commonly the rat. Although at times such growth has been broken down into its components, as in the case of calcium deposition, or extended, as in that of reproductive capacity, the information so gained is inherently limited in general biological terms. Similarly, studies on microörganisms customarily are made in terms of population growth rates and densities, although in isolated instances some particular phase of development, such as sporulation in certain fungi, has been made use of as a criterion.

The manner in which the response of the experimental animal is to be measured is frequently a purely technical matter, the animal itself imposing the practical limitations. Considerable advantage would appear to be gained, therefore, by the use of an experimental animal which allows the assay of a wide variety of responses. From this point of view, certain insects are particularly suitable for such investigations because they can be studied in terms of the development or survival of individuals or populations; their life-history is frequently complex in that they go through successive developmental stages; and last, but not least, not only may fertility be studied but it is differentiable from fecundity. Therefore, we have undertaken a study of the nutritive requirements of the flour beetle *Tribolium confusum* Duval, an insect which has been studied by one of us (T. P.) for a number of years in connection with certain population problems. The purpose of the work reported in the present paper is to lay a foundation for the later development of a "synthetic" nutrient medium of definite chemical composition, an obvious prerequisite to any precise definition of nutritive requirements.

In general, the nutritional aspects of insect physiology are not too well known; such studies as have been made are reviewed by Hoskins and Craig (1935) and by Wigglesworth (1939). *Tribolium confusum* is readily grown in the laboratory in the usual commercial flours, and some of the environmental factors affecting its growth in such media

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have been considered in a recent review (Park, 1941). Studies on the nutritive requirements of this beetle were initiated by Chapman (1924), who used a medium consisting of cornstarch, various proteins, inorganic salts, and wheat germ. Growth was assayed in terms of larval head size, since the latter is directly related to the number of molts, and of the development of beetle populations. The importance of wheat germ was apparent, and in general it seemed that requirements for growth in terms of increasing head size were much less exacting than those for maturity or transformation. Sweetman and Palmer (1928) assayed similar culture media in terms of the length of the larval period and, for this developmental stage, could demonstrate no need for vitamin A; but, in confirmation of Chapman, the undifferentiated vitamin B complex appeared to be necessary. Nelson and Palmer (1935) used various rations, including Farina and similar materials, and, in addition, a mixture of arachnin, edestin, Crisco, inorganic salts, and a 50 per cent alcoholic extract of yeast. No need for vitamin D was observed, but the time of pupation appeared to be related to the amount of phosphorus present, the limiting amount being approximately 0.1 per cent. These workers accounted for the slower development and smaller populations of *Tribolium* observed in patent flours and similar products by the low phosphorus content of these foods. Street and Palmer (1935) observed that the flour beetle requires not only vitamin B_1 but an additional heat-stable factor present in autoclaved yeast. The stimulating effect of yeast in terms of larval production and rate of larval growth and on population densities was also observed by Bushnell (1938) in studies with yellow corn meal. A higher percentage of individuals was found to survive the metamorphic period when reared in corn-meal–yeast medium than when reared in corn meal alone. The report of Chiu and McCay (1939) likewise indicated a need for vitamin B_1 and a heat-stable factor present in yeast and, as well, the dispensability of the fat-soluble vitamins A and D when the beetles were grown on a purified diet consisting of casein, cornstarch, yeast, cotton seed oil, and inorganic salts. There was evidence of the inability of the beetles to utilize sucrose. These experiments were based on the growth and survival of beetle populations—the number found in 49- and 94-day cultures originally inoculated with ten newly emerged adults. It has also been shown by Lund and Bushnell (1939) that yeast exerts a stimulating effect on fecundity in patent-flour cultures. More recently Fraenkel and Blewett (1941) reported that larval development was accelerated in highly milled patent flour by the addition of vitamin B_2 (riboflavin) but not by that of pure vitamin B_1 (thiamin, aneurin). Barton-Wright (1941), however, pointed out that either riboflavin or nicotinic acid produces such an acceleration and that a specific riboflavin deficiency in patent flour, and therefore nutritive requirement, was not demonstrated by Fraenkel and Blewett. He presented further experimental evidence corroborating the results obtained by earlier workers with respect to the necessity for vitamin B_1 and an, as yet, unknown heat-stable factor present in yeast.

It appears, therefore, that the nutritive requirements of *Tribolium* are satisfied by a relatively simple diet consisting of purified protein and carbohydrate, of inorganic salts, including phosphates, and of yeast or certain fractions of yeast. Because of the diversity of methods of assay, a more definitive general statement is not, as yet, possible.

EXPERIMENTAL

Preparation of the medium.—The studies reported here are similar in a general way to those of other workers. Two points are to be noted, however. First, a nonnutritive

diluent, wood dust, constituted the bulk of the medium. Second, the response of the beetles to this environment was assayed, for the most part, as fecundity, i.e., the number of eggs laid, and as fertility, i.e., the proportion of the eggs which proved to be fertile. The ability of beetle populations to maintain themselves in the experimental medium is considered here only briefly, although in a definitive study this would be an essential aspect.

The medium developed for use in these experiments contained the following substances:

Wood dust	81 gm.	Casein	10 gm.
Osborne-Mendel salt mixture	1	Dry yeast	5
Dextrose	3		

The physical state of this medium proved to be of great importance. Preliminary experiments in which cornstarch was used as a diluent were completely unsatisfactory in that the normal tunneling operations of the beetles were greatly restricted and imagoes did not survive long, possibly owing to asphyxiation. Other experiments with a fine sawdust, or wood dust from a sanding machine, showed that this substance was satisfactory; the activity of the beetles appeared to be much the same as in the usual flours. It was observed, however, that wood dusts differ from one another with regard to the shape of the particles, a consequence no doubt of the milling operation involved, though this was not definitely ascertained. In any case, a granular type of dust seemed much superior, for present purposes, to a fibrous type containing many long fibers of wood. We attribute the less satisfactory experimental results reported here to the use of the latter type of wood dust, although we can offer no definitive evidence on this point.

In all the experiments reported here a pine-wood dust was used and the resinous material removed by extraction for 6 hours with hot alcohol. The alcohol was filtered off on a Buchner funnel, and the dust was washed with successive portions of hot alcohol. The alcohol was removed by drying in large evaporating dishes in an oven at 85° C. for 10–12 hours. The dry dust was sifted and resifted through a silk bolting-cloth sieve having 82 meshes to the inch.

The Osborne-Mendel salt mixture was that supplied by the scientific houses for nutrition experiments. The dextrose was Pfanstiehl's "C. P. Anhydrous." We have used both vitamin-free casein and a purified casein designated as Pfanstiehl's "Casein, Pure" and have not detected any difference in them in these experiments. The yeast was Fleischmann's dry brewers' yeast.

The nutritive ingredients were ground in a ball mill (the yeast need not be ground) and sifted through the 82-mesh sieve. It is essential that the entire medium pass readily through such a sieve so that it may be separated from the eggs for fertility and fecundity studies. These substances were then mixed in the proper proportions with wood dust, first by grinding for a few minutes in a mortar with wood dust (if this is not done, difficulty is experienced in securing a uniform mixture, for the finely ground casein and dextrose in particular tend to remain aggregated), followed by mixing the entire batch of medium in a ball mill running at a reduced speed (*ca.* 30 r.p.m.) without the balls. A mixing period of 2–3 hours appeared to be adequate for uniform dispersal of the ingredients; analyses for glucose on samples taken at random from such mixtures gave values within the limits of error of the analytical method.

The moisture content of this medium is very low at this point because of the dehydration of the wood dust by the alcohol extraction. As will appear, in some of these experi-

ments the medium was also heated to 85° C. for 10–12 hours after mixing. In any case, additional moisture was required. The relation of the growth of *Tribolium* to moisture is known largely in terms of humidity of the incubator air rather than in moisture content of the medium itself. The water content of the flour media under satisfactory incubator conditions was found to be 11–12 per cent. The dry wood-dust medium acquired about this moisture content when exposed, in layers 2–3 cm. deep, to a water-vapor atmosphere at room temperature (attained in the ordinary vacuum desiccator containing water and evacuated).

This medium does not keep indefinitely, for mold growth appears in the course of a few weeks. In these experiments batches of medium were freshly prepared within 2–3 days of use.

Experimental procedure.—The humidified medium was distributed in shell vials, 95 × 25 mm., in quantities of 3 gm. each. At the beginning of each experiment one pair

TABLE 1

BIOMETRIC CONSTANTS FOR FECUNDITY

Experiment	Mean No. of Eggs per 5-Day Period	Standard Deviation (Eggs)	Coefficient of Variability (Per Cent)	*n*	Medium	Parasite-Free
B	22.3±0.67	9.7±0.47	43.5± 2.11	96	Wood-dust medium	No
C	11.2±0.52	9.6±0.35	85.7± 3.29	154		No
D	9.4±0.35	8.6±0.25	91.5± 2.61	280		Yes
H	14.1±0.33	8.2±0.23	58.1± 1.65	280		Yes
I	3.6±0.53	6.1±0.37	169.4±10.43	60		Yes
J	16.8±0.72	8.3±0.51	49.4± 3.04	60		Yes
K	18.1±0.50	12.5±0.35	69.1± 1.96	280		Yes
F	29.9±0.83	19.1±0.59	63.9± 1.96	240	Ceresota flour	Yes
A	2.5 (20 days)	.....	.....	8	Wood-dust control	No
E	0 (15 days)	.....	.....	40		Yes

of just emerged imagoes of closely inbred stock was placed in each vial. The cultures were maintained in the dark at 29° C. in a relative humidity of 65–75 per cent. At 5-day intervals the medium was sifted, the eggs counted and retained for fertility studies, and the pair transferred to a vial of fresh medium. The beetles were removed before sifting in order to reduce handling as much as possible. Although a variety of preliminary experiments were carried out with as few as 10 pairs of beetles, those reported here involved 30–40 pairs. Analyses for glucose indicated that, though this substance was used up by the beetles, it was not exhausted in the 5-day period; concentrations never dropped below 2 per cent. Control experiments with flour and with wood dust containing no added nutriment were, of course, run. The results of these experiments in terms of fecundity are recorded in Table 1 and Figure 1.

From these data the following points are apparent:

1. With regard to mean fecundity, there are differences between certain of the experiments that are greater than can be expected on a chance basis alone.

2. The mean fecundity of the flour controls is significantly higher than that of any of the wood-dust media.

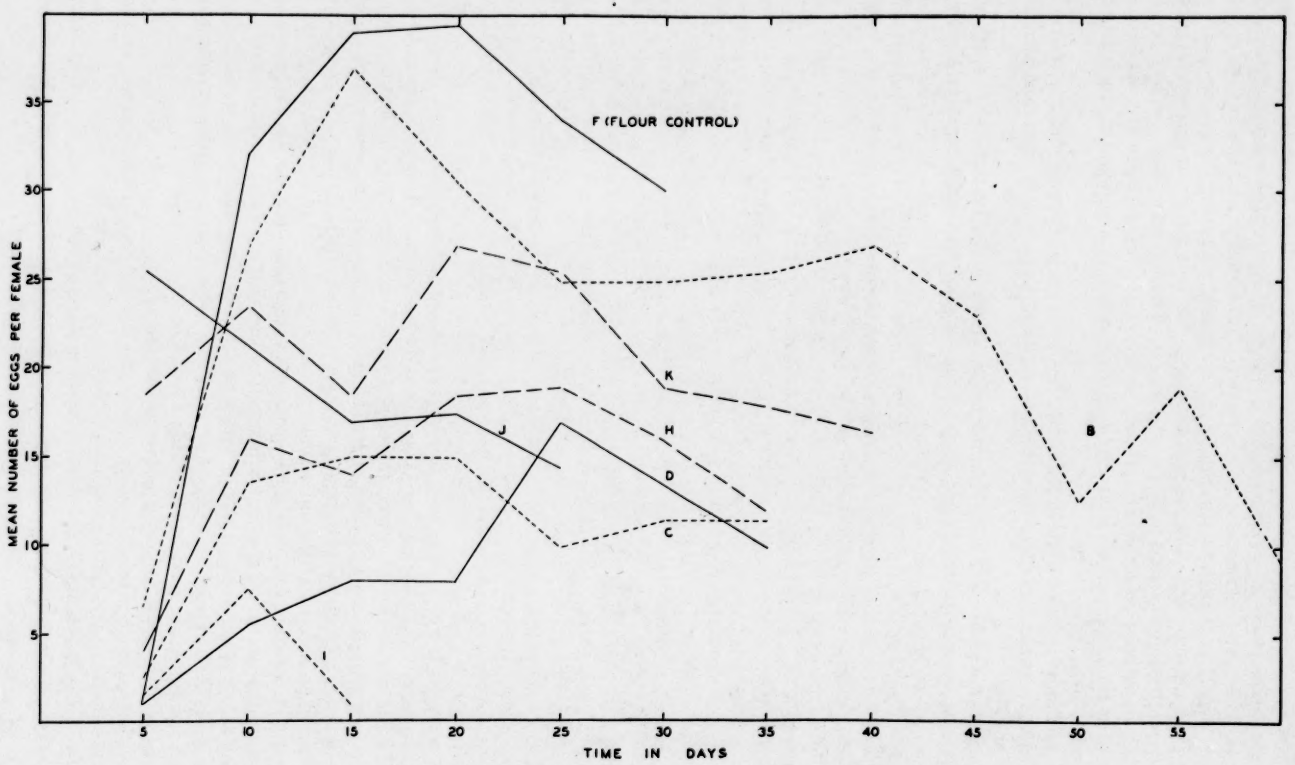

FIG. 1.—Fecundity of flour control and wood-dust experimentals

3. *Tribolium* kept in wood dust without added nutriment produced a negligible number of eggs and died within the first 20 days of imaginal life.

4. With regard to the variability of oviposition, two points can be made: (*a*) In certain of the series, including the flour control (especially C, D, H, K, and F), the variation, as indexed by the coefficient of variability, is somewhat higher than that reported in earlier studies (Park, 1935; Park and Woollcott, 1937). We have no explanation of this point at the moment. (*b*) There is considerable variation both in degree and in pattern between individual experiments. This point is illustrated in Figure 2, where variability is plotted on the abscissa as percentage deviations from the mean on a percentage frequency ordinate.

It is also apparent that a sharp drop in fecundity occurred after the first experiment, B series, rising again in the last experiments. A possible explanation was thought to lie in a chronic infection with *Adelina triboli* common to *Tribolium* stocks (Bhatia, 1937). Only the experiments of series B and C were carried on with such infected stock. In the remainder (including the flour control, F) the infection was eliminated by immersing the eggs in a 1:1,000 solution of mercuric chloride for periods of 2–10 minutes, followed by successive washings with sterile water on a small sterilized Buchner funnel. It has been shown histologically that such treatment is effective in removing the parasites. Culture of such eggs and the larvae hatching from them in the usual nutrient-broth bacteriological culture medium indicated that in all cases the beetles were bacteriologically sterile also. No attempt was made to carry bacteriologically sterile stocks, however; but these stocks were carried separately and in heated media to avoid contamination with the parasite. The possibility of working with bacteriologically sterile *Tribolium* itself offers some enticing areas of research.

Table 2 lists the mean fertility percentages for those experiments (D, H, K) and the control (F) where eggs were set aside and larval counts made. This fertility performance is quite in line with the general results of earlier work on *Tribolium*, with regard to both the level and the stability of fertility. In general, it has been the experience that, while fecundity is highly susceptible to ecological modification, fertility is quite stable biologically and not easily modified. In the light of this it is somewhat surprising to note that the beetles of experiments D, H, and K had a higher mean fertility than those of the flour control. This difference is significant; it is, in fact, 9.4 times its standard error. This suggests, therefore, that in some way the growing of beetles in the wood-dust medium increases their fertility while decreasing their fecundity. We do not wish to advance this as anything beyond a tentative suggestion for the present; the point requires additional evidence before it can be considered as established. However, we do feel that this ability to break down reproduction illustrates one experimental advantage that insects offer over certain other forms.

In addition to the study of fecundity and fertility, small stock cultures of *Tribolium* were started in the wood-dust medium. These cultures maintained themselves for several generations without changing or replenishing the environment. It should be emphasized that these stocks did not build up to the population densities that *Tribolium* normally produces in white flour. This is to be expected in part on the basis of lowered rate of oviposition of the beetles in the wood medium, though probably other factors are operative also.

As pointed out above, it is evident that there is considerable variation between individual experiments with the wood-dust medium. We can offer no satisfactory explana-

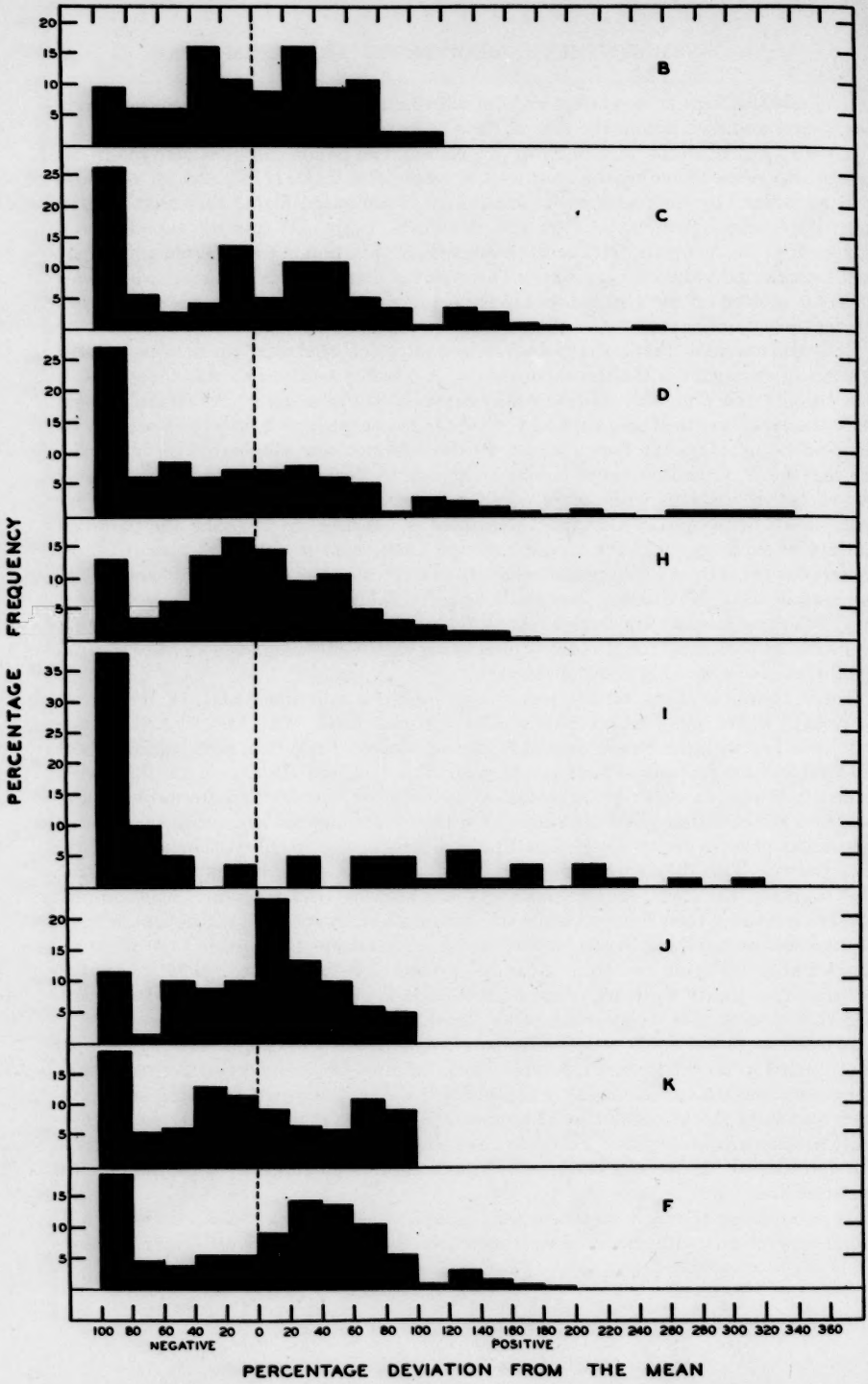

FIG. 2.—Variability in egg production expressed as percentage deviation from the mean

tion of this variability. Apparently, it is not a consequence of infection with *Adelina* (at least to the degree prevalent in our beetles), since the B series is quite as good or even slightly better than the J and K series; the last two were free from infection, while the first was not. Similarly, there is a relatively low egg production in series C, D, H, and I; and here only C was infected. It seems more probable that these differences between the difference series of experiments are associated with the physical state of the medium in that in series B, J, and K a granular type of wood dust was used, while in the other series the fibrous type was used.

TABLE 2

PERCENTAGE OF EGG FERTILITY

Experiment	Medium	Time in Days							Mean No. of Larvae	Total No. of Eggs	Total No. of Larvae	Percentage of Fertile Eggs
		5	10	15	20	25	30	35				
D.....	Wood dust	66.7	74.9	68.2	80.8	76.5	77.3	69.8	74.8			
H.....	Wood dust	68.5	67.4	74.5	77.5	79.3	79.9	82.1	76.4	10,374	7,624	73.5
K.....	Wood dust	.....	70.0	69.6	69.3	82.3	62.2	75.7	69.6			
Mean.....		67.6	70.8	70.8	75.9	79.4	73.1	75.9	73.5	.....	.....	.....
F.....	Flour control	54.1	74.6	68.0	61.4	69.0	64.8	.....	67.0	7,174	4,815	67.0

DISCUSSION

The medium described here appears to constitute an adequate, though by no means optimal, culture medium for the flour beetle. That it is nutritionally adequate is indicated by both continuous and regular oviposition over periods of observation as long as 60 days and the ability of the beetles to maintain themselves as a stock population in this medium over a period of months. Although questions such as metamorphosis, rate of larval growth, time of pupation, physical development of the metamorphic stages, and the like have not been studied per se here, the beetle does complete and repeat its entire life-history in this medium. It is highly improbable that the wood dust constitutes a nutrient for the beetles; in fact, they lay few eggs and die off rapidly in wood dust alone. These experiments show, then, that a diet of purified casein, a mixture of inorganic salts, dextrose, and yeast satisfies their nutritive requirements. That it is not optimal, however, is indicated by the unfavorable way in which it compares with flour with respect to both rate of oviposition and population densities maintained in stock cultures.

It is to be emphasized that the wood-dust medium, physically satisfactory and nutritionally adequate, serves as a point of departure for the experimental demonstration of the more precise nutritive requirements of these organisms with respect not only to vitamins but also to nitrogenous compounds and energy metabolism, as measured in terms of fecundity, fertility, and a variety of other responses not touched upon in the present paper.

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SOME EFFECTS OF CONDITIONING ON SOCIAL DOMINANCE AND SUBORDINATION IN INBRED STRAINS OF MICE

(One figure)

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DOMINANCE-SUBORDINATION patterns of behavior, such as the so-called "peck-orders" in birds, have been demonstrated for many different groups of vertebrates ranging from fish to man. Interest in the subject now centers mainly on inquiries concerning the extent to which such social organizations exist in nature and upon an analysis of the factors which underlie and control their establishment and maintenance.

Although this type of group organization exists under natural (Odum, 1941) and seminatural conditions (Lorenz, 1935; Jenkins, in press), it has been most studied with caged or penned animals. In these kept groups, individual social status is achieved either by fighting or by the passive acceptance of subordination. The relative social position of any two individuals with regard to each other may be determined largely by the result of their first pair-contacts, as with hens (Schjelderup-Ebbe, 1922); or there may be a long series of such contacts during which dominance within the pair shifts back and forth before stability appears. Under certain conditions and with some animals, dominance never becomes absolute, and the social flock is organized on a give-and-take basis in which the relative social status of any given individual is judged by the ratio of its wins and losses with each member of the flock.

One of us has recently reviewed, in a general way, present knowledge concerning the factors that make for social dominance (Allee, 1942*a*, 1942*b*), and we shall return to the subject in the final section of the present paper. The factors include, among others, heredity, on the one hand, and previous experience or conditioning, on the other. In this paper we are concerned mainly with an analysis of some phases of the effect of conditioning on social status. More specifically, the question we asked ourselves and these animals was: Does a recent victory or series of victories tend to condition further successes, and, on the other hand, does defeat tend to produce further defeats? There were indications in the literature (cf. Whitman, 1919, with doves; Uhrich, 1940, with mice; Diebschlag, 1941, with pigeons) and in the experience of one of us with hens (Allee and Collias, 1940) which indicated that an affirmative answer might be expected, providing we were able to secure proper experimental material and to set up appropriate experimental conditions.[1]

[1] Hall and Klein (1942), in a paper which appeared while the present article was in press, concluded from their experiments that (1) "there are marked differences in aggressiveness between rats"; (2) "aggressiveness appears to be a fairly stable and consistent trait"; and (3) "rats selectively bred for fearlessness in a strange situation were found to be more aggressive than rats selectively bred for timidity." They did not test for the possibility that aggressiveness might be affected by conditioning. As with our mice, body weight and the home cage were not important correlates with aggressiveness in these rats.

Material and Methods

Mice were selected for initial experimentation, in part because male mice are notably combative. Among others, Crew and Mirskaia (1931), Retzlaff (1938), and Greenwood *et al.* (1936) have found that fighting is an important cause of death in groups of laboratory mice. In this connection the last-named authors say: "Many, perhaps most, of the deaths in a herd of normal mice are due to fighting, and it would seem that an immigrant requires a few weeks to obtain recognition as a member of the herd to whom average toleration must be extended."

Uhrich (1938) worked for more than 2 years in this laboratory upon the social order among inbred albino mice. As a result of the study of group relations among some 300 male and 150 female mice, he reported critical evidence which showed that small groups of mice, when caged together, exhibited a social order. For the most part, the order was based on the exclusive dominance of a single mouse which was superior to a number of subordinates that were approximately equal in position. Various other orders also existed for longer or shorter times—among them, a linear order such as is characteristic of the peck-order in hens.

Uhrich also concluded (1938, p. 402) that, despite evidence that mice may at times react to another mouse as an individual, "it sometimes appears as though the subordinates do not recognize the dominant [as an individual] but merely flee from any mouse that happens to attack them or assumes a threatening attitude." A social order based on an impersonal recognition of belligerency, real or threatened, seemed more suitable for our experimentation than did one based on recognition of and reaction to individuals as such.

Later Uhrich (1940) combed through his records and found suggestive evidence that the previous fighting history gave some indication of the outcome of future fights. Briefly summarized, this evidence was that, with mice of known fighting history, some individuals with 12 defeats to their joint credit lost 9 and won only 3 of their next 12 fights. Conversely, another set with 32 previous victories to their joint credit won 20 times in their next 32 starts. Uhrich himself recognized the imperfect nature of this evidence. Its greatest weakness lay in the lack of discrimination between native belligerency of the individual mice and a possible conditioning effect of their preceding experience.

With mice such as Uhrich had been using, a successful attack upon this phase of the problem would be tedious. We were helped at this point by the discovery of Scott (1940), who found in his study of prefighting behavior that different inbred strains of mice at the Jackson Memorial Laboratory show clear-cut genetic differences in aggressiveness. These results suggested that we could use such genetic strains and have predictable outcomes of at least some of the interstrain fights. This would enable us to carry on conditioning and later to test the effects of such experience by rematching with other members of the same strain.

Miss Catherine Fales made a preliminary test of the possibilities as a class project in animal behavior and found that it was possible to cause previously superior or equal mice to become subordinate after a series of 15 successive defeats and that inferior mice tended to fight sooner when their former superiors had been defeated repeatedly. At this point Miss Fales decided to leave the problem, and we took up active experimentation with the mice which she had been using.

At the beginning we had available C-57 blacks (aa), C-3H agoutis (wild type), and C (Bagg) albinos (bbcc), which had been given us by the Jackson Memorial Laboratory,

to which we hereby express our thanks. In each case these animals were from two litters and were between 41 and 47 days of age upon arrival at this laboratory about 2 months before the start of the present series of experiments. In addition, we had several litters of the so-called P(aabbddpps$_e$s$_e$) stock and several C albino animals of a different substrain which were obtained from Dr. E. L. Green. We also caught some random-bred wild mice in live traps in the laboratory. We worked seriously with the first three stocks only.

All the animals in the first three strains had been bred through at least fifteen generations of brother-sister matings. For fuller descriptions of the stocks and for a summary of the effects of inbreeding see the stock lists of the Jackson Laboratory and the account of Russell (1941).

In all, we observed 110 male mice from three generations. Males only were used in the actual fighting. Unless otherwise specified in comparable tests, the animals were litter mates or were the offspring of litter mates born a few days apart. All animals were kept in a well-ventilated darkroom. The temperature was held between 20° and 23° C., except during experiments designed to test the effects of more extreme temperatures upon fighting.

Unless otherwise stated, each mouse was housed separately in a coarse-meshed wire cage 9.5×9.5×7 inches. Each cage was equipped with a water bottle and a sufficient amount of cotton and excelsior to allow nests to be built. The animals were fed Purina Fox Chow Checkers in excess of the amount they would consume daily. Cages were cleaned once each week.

Combats were carried on in neutral wire cages ($\frac{5}{16}$-inch mesh) with metal bottoms. They measured 9.5×9.5×12 inches and were divided by a galvanized iron partition which could be readily raised. A mouse was transferred to the fighting cage and back to his home cage in the box in which he was routinely weighed. This procedure did not seem to excite the mice as much as manual handling, and it took considerably less time than when the home cage and the fighting pen were brought together for the transfer. Experiments with box transfer and direct transfer from the pen gave the same results. Fights were started by raising the partition between the two mice.

During a combat, ceiling lights were turned off. The fighting cage was removed some 10–12 feet from the home cages. A 60-watt lamp was placed about 7 inches from the top of the cage. This hindered the mice from seeing outside and, particularly, from reacting to the observer. The near-by light raised the temperature within the cage about $\frac{1}{2}$° C. Unless the fights became too severe, they were allowed to run for 20 minutes, longer on occasion.

The mice were weighed from time to time, and, with generations reared in our laboratory, records of growth in the prefighting age were also taken.

Observations

The pattern of activity which constituted a typical fight was as follows:

1. Mutual sniffing
2. Independent activity, exploration of the cage, accompanied by fluffing of the hair and rapid breathing
3. Lashing of the tail
4. *a*) An attempt to bite back when sniffed at (more common in first encounters)
   b) Direct attack by one member of the pair (common after conditioning)

5. A series of hard and rapid attacks and counterattacks
6. Intermittent chasing and biting
7. The chased mouse rears on its hind legs, draws one foreleg close to the body, extends the other stiffly, remains motionless, and squeals when touched by the other mouse

Point 7 will hereafter be referred to as "submission" and is one important criterion used to judge active or passive defeat. Significant variations from the above pattern will be mentioned separately. The position of the forelegs during "submission" was decidedly different from the poised stand which was frequently assumed just before an attack.

INTERSTRAIN DIFFERENCES

In order to provide a basis for testing the effect of successive wins and losses on the outcome of future battles, it was necessary to assay the differences between the strains. If these were consistent, then conditioning, if effective, should produce reversals in the order of interstrain dominance. The results of calibrating tests are recorded in Table 1.

TABLE 1

A SUMMARY OF THE OUTCOME OF PRELIMINARY TESTS OF THE RELATIVE FIGHTING ABILITY OF THREE STRAINS OF MICE

Winner	C-57 black	C-3H agouti	C-57 black
Loser	C-3H agouti	C albino	C albino
Number of animals	10	10	10
Number of battles	133	137	144
Percentage of victories:			
Active	66.2	40.9	61.1
Passive	9.8	24.8	4.2
Percentage of "no aggression"	18.8	24.1	31.9
Percentage of defeats:			
Active	0.0	4.4	0.0
Passive	5.2	2.2	2.8
Percentage of draws	0.0	3.6	0.0

Active defeats and victories are determined by battle, while passive ones result from submission of one animal without fighting. It is evident that, whenever a fight took place, the blacks were clearly superior to the other strains and the agoutis were superior to the albinos. In some cases the agoutis displayed more aggressive behavior prior to the actual fight than did the blacks.

All these mice had previously been fought under the supervision of Miss Fales (1940), who had obtained similar results. A period of isolation of 3 weeks' duration had ensued. The first 5 fights between the blacks and agoutis were initial contests for both strains during the present experimental period. The contests were subsequently extended until every mouse had met every other mouse, round-robin fashion, in order to detect individual upsets if any should occur; none did. The behavior of one mouse (Bl_3) ran counter to the expected trend in that it showed no aggressiveness during any of its encounters. This animal had been very badly beaten during the experiments made by Miss Fales and, as will be shown later, had probably been conditioned so that it showed aberrant behavior as compared with other members of its strain.

In the agouti-albino series the albinos may have been favored by the fact that the agoutis had recently been defeated by the blacks. The agoutis, however, proved to be

consistently superior; and, characteristically, the albinos put up a series of poor defenses against the more agressive animals.

The P stock was erratic and highly variable in fighting behavior and was not used in this group of experiments. Perhaps the large number of mutations carried by this stock influenced its vigor and behavior, or, more likely, the fact that it was less highly inbred may account for these variable results.

The data given in Table 1 indicate that there are distinct behavior differences between the three strains tested and that, under our conditions and treatment, the blacks were most successful, the agoutis were less so, while the albinos were most pacific. We thought it wise to retest these relationships with mice which we had reared and whose early experience had been carefully controlled and observed. Accordingly, a second series of 88 interstrain contests were staged between each of the three strains; the animals used had been isolated after weaning. The results will not be summarized in detail, since they support the data already given. The only major deviation is that these younger, less experienced mice showed more instances in which no fight resulted from placing two males together.

Another similar series was arranged which consisted of 54 fights between second-generation blacks and agoutis. This time the young mice were reared with their litter mates until transferred to the fighting cage for the first battle. Again the results are in entire agreement with those already described. No effect of communal rearing was noted other than a possible tendency for the mice to be less excited in the presence of another individual.

As another variant, litters borne by black and by albino females on the same day were split shortly after birth, so that each dam reared both black and albino offspring together. Some of these (*a*) were isolated after weaning and were kept so until they were mature enough to fight; others (*b*) were kept together with the same number of males in each lot; the number of females differed with each group. They were removed as soon as it was possible to determine sex accurately. We were also able to make exploratory tests of interstrain fighting between individuals (*c*) that had the same foster-mother and to compare the results with the outcome of other fights (*d*) in which the foster-mothers had been different. The numbers available for these tests were as follows: (*a*) 4; (*b*) 4; (*c*) 2; (*d*) 2.

In all these cases the result was still the same as has already been reported; and, while the numbers are too small to allow a final statement, there was no indication of differences in the outcome of interstrain fighting which could be related to early experience. It will be remembered that we attempted to allow these fights to continue until there had been an actual defeat. This means that our criterion of success combines the factors of aggressiveness in starting a fight with that of success in fighting. Table 2 summarizes the results from all these preliminary interstrain contests between mice that had been reared in our laboratory.

On the basis of the statistical data presented in Tables 1 and 2 we conclude that, in our hands and under the described conditions, the C-57 black mice used in this study were definitely superior to the C-3H agoutis and the C albinos and that the latter were inferior to the agoutis in ability to win fights. The mice were assorted according to strains in their first few fights. Other observations, which are not readily summarized statistically, support these conclusions. Consistent changes in this order which are correlated with

conditioning may, if environmental conditions are kept constant, be ascribed to conditioning.

Differences other than superiority and inferiority in fighting were also observed between the strains. These will not be reported in detail. So far as they go, however, they tend to support the hypothesis advanced by Scott (1942) and others that there is a genetic basis for such traits. It is pertinent to our main interest to report that the black males were more sensitive to handling and were more easily made highly nervous than were the males of the other strains studied. Unless the blacks were handled gently, they often failed to fight when placed with a stranger, and they squealed every time the cage was moved. Similar treatment did not cause as severe a response on the part of any of the males of the other strains.

Agouti males started fights most frequently in initial encounters, but chiefly by feinting or nipping when sniffed at. Neither the blacks nor the albinos were so likely to make false feints and lunges as were the agoutis.

TABLE 2

THE RESULTS OF FURTHER TESTS OF THE RELATIVE FIGHTING ABILITY OF THREE STRAINS OF MICE

Winner	C-57 black	C-3H	C-57 black
Loser	C-3H agouti	C albino	C albino
Number of animals	14	8	20
Number of battles	142	88	208
Percentage of victories:			
Active	61.3	40.9	33.9
Passive	10.5	21.6	25.4
Percentage of "no aggression"	28.2	37.5	40.7

It is evident from the data that not every initial meeting of two male mice results in a battle. There may be much random behavior, mutual sniffing and grooming, and no display of aggressiveness. Females are regularly less aggressive than the males; and, while they will fight between themselves, such fights are more rare than when two males are concerned. What, then, are the immediate causes of a fight between males?

It is impossible to give a satisfactory answer on the basis of observed battles. There is, however, one conjecture which is suggested after watching even a few fights. This suggestion is that initial fights develop on occasion from a modification of normal sex behavior. Strange males that have been kept in isolation and are meeting another male for the first time since weaning begin to sniff and nudge him as though he were a female. This is not long sustained, since such behavior is usually sufficient cause for the other mouse to nip at the more aggressive individual, and a fight commonly ensues.

Where mice have had previous fighting experience, such exploratory behavior is usually omitted. An experienced, aggressive male, when he meets another male, shows increased activity, fluffs his hair, thrashes his tail, and exhibits other phases of "threatening" behavior. If an experienced male is pacific, he tends to keep to himself or tries to groom the other mouse. In certain other circles we would say that he was either an isolationist or an appeaser.

If an unaggressive male is allowed to copulate and is then placed with another male, which may or may not be aggressive, the second pursues the first rather insistently

(probably attracted by odors incident to copulation). This method has been used in these experiments in attempts to provoke fights between nonagressive animals and was somewhat successful.

INTRASTRAIN DIFFERENCES

In addition to knowing the interstrain differences in fighting ability, it was essential to find the intrastrain status of each individual mouse. Accordingly, an extended series of intrastrain fights were staged in order to "calibrate" every mature male mouse in the colony against the other mature males of his own strain and general age class. With such information at hand we could then detect relatively subtle changes in the order of dominance which might arise during the process of conditioning.

For these intrastrain contests the mice were treated exactly as they were for the interstrain battles except that now the members of each strain were fought against each other every fourth day until a fairly stable order of dominance was reached. Care was taken to fight the mice in a different order in each successive round.

THE C ALBINO STRAIN

The first series of staged pair-contacts between a lot of five males of this strain will serve to illustrate in some detail the characteristic behavior of this particular stock. Each set of symbols—W_1, for example—represents a single animal.

W_1 *vs.* W_2: The mice were transferred to the fighting cage and left in separate compartments until they showed no signs of undue excitement. The partition was then rattled and lifted. Each mouse ran into the half of the cage which was newly opened to it. For the next 3 minutes each explored the cage independently. Then they touched vibrissae in passing. Mutual sniffing followed, and they subsequently groomed each other. There was no evidence of hostility or fear. At the end of 20 minutes the partition was lowered, and the animals were transferred to their home cages. The encounter was recorded as "no battle."

The behavior of the following contact-pairs in their initial meeting in this series did not differ significantly from that of the pair just described: W_3 *vs.* W_5; W_6 *vs.* W_1; W_3 *vs.* W_1; W_5 *vs.* W_1.

W_2 *vs.* W_3: W_3 rattled its tail once during the 20-minute period. There was no other sign of hostile activity.

W_5 *vs.* W_6: After 5 minutes of typical random behavior, W_6 nipped at W_5, which immediately squealed. W_6 then attacked, and W_5 gave a typical submission reaction. The entire conflict lasted 17 seconds.

W_2 *vs.* W_5: After 1 minute of random behavior W_2 attacked, and W_5 submitted at once.

W_3 *vs.* W_6: As soon as the partition was lifted, both animals showed signs of excitation. Their breathing was rapid and labored; the dorsal hair of each was erected; movements were quick and jerky; and both mice frequently rattled their tails in passing each other. W_6 attacked, and a series of spirited skirmishes followed. Each attack was fast and vicious. Single battles lasted up to 46 seconds and were followed by periods of from ½ to 2 minutes of independent activity. After 7 minutes of such behavior, each mouse retired to a separate corner and groomed itself. Then they began all over again. After 11 minutes each retired to his previously occupied corner, but this time they both lay prone on the bottom of the cage for 3 minutes in seeming exhaustion. Another series of battles

followed. At the end of 20 minutes neither mouse had shown signs of submission, and the encounter was recorded as a draw.

W_2 *vs.* W_6: W_6 showed typical signs of aggressiveness (hair-fluffing, tail-beating, etc.) and attacked almost immediately. W_2 made no attempt to fight back but squealed at each attack. After 9 minutes of such behavior, W_2 gave the submission reaction and the fight was stopped. W_6 was, of course, recorded as the winner.

Each mouse had its own peculiar fighting habits, and changes in relative aggressiveness between the members of a contact-pair were easily observed. As the series of battles progressed, some mice (notably W_5) submitted passively before being attacked. Such passive submission was usually to an aggressive, and almost never to a pacific, mouse. Aggressive mice from another substrain of albinos, which did not differ noticeably in behavior from those most used, were introduced from time to time in an effort to determine whether the submissive animal "remembered" a specific mouse by which it had formerly been beaten or whether it reacted to the general deportment of an aggressive individual. The latter seemed to be the case. Further evidence on this point is furnished by the behavior of these mice when matched against the aggressive black strain. Under these conditions the albinos did not usually submit to Bl_3, which never attacked, but did submit passively to all the other blacks. They did not, therefore, react to the strain color, or to a possible strain odor; and we have no evidence that they remembered individuality. The whole behavior complex can be adequately interpreted on the supposition that these mice were responding to the deportment and activity of their opponents in the fighting cage.

For the purpose of ranking the animals a victory following a fight was considered more important than a victory by passive submission, since the most aggressive animals attacked regardless of what the other mouse did. The lack of any fighting within the 20-minute period was taken as an indication of at least temporary pacifism, and continued lack of such fighting was thought to reveal a generally pacific or passive "character."

The most aggressive mice (as already noted) typically attacked opponents almost immediately. The least aggressive typically submitted passively almost as soon as the partition had been lifted and the presence of an opponent was sensed. Mice in the middle of the order seemed to watch for behavior clues; they attacked, if at all, only after some time had elapsed, and they seldom submitted until an aggressive opponent actually began his attack.

Figure 1 summarizes the observed changes in the order of dominance during a series of 200 intrastrain fights among the albinos. The order of dominance is indicated by the ordinates, and the number of fights which had occurred in this series is shown by the abscissae. At times two or more individuals occupied the same rank.

The available data indicate that the order of dominance is not established by the initial encounters and that a relatively stable order emerges only after a considerable number of battles. Fluctuations at the top and the bottom of the social order are more rare than are changes in the mid-ranks.

The system of obtaining social rank by considering the number of active and passive victories and defeats was tested after 130 such pair-contacts had been staged. All five of the albinos were placed in the fighting cage simultaneously. Under these conditions W_1 was dominant and W_5 was most subordinate. At the end of 200 fights the others were intermediate in about the same way in which they are shown in Figure 1. The

ranking system, as used, was accordingly judged to be a fairly accurate index of the true order in group dominance.

Mouse W_1 showed no agressiveness in his initial battles. He fought back when attacked and, at first, did not pursue the attacker after he had been driven off. Evidently prefighting aggressiveness in unconditioned mice is not necessarily correlated with success in fighting. As the series progressed, W_1 became more aggressive and at length became and remained the alpha-mouse of this lot. W_2 showed similar tendencies but was not so successful as W_1. After a series of 7 defeats by the latter, W_2 dropped to a position of parity with W_3 and W_6.

The changes in aggressiveness of W_3 and W_6 after 50 or 60 contact reactions with the other mice of this lot did not seem to be a result of actual defeats but rather of the long series of hard-fought battles. This is an exception to our general experience. Both of these mice were relatively aggressive as compared with other members of this strain; they fought longer and harder than any of the other albinos. They were never defeated with the same decisiveness with which they defeated their opponents; yet, after a few relatively mild defeats, both mice began to lose status, which they did not regain. This drop was sharply paralleled by increased aggressiveness on the part of W_1 and W_2 and by reduced passiveness on the part of W_5 when opposed either by W_6 or by W_3. Even relatively subtle changes in attitude are apparently detected by other mice when the animals in question have frequent contact.

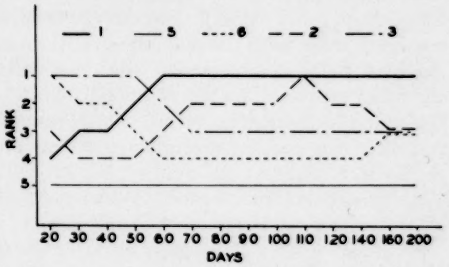

FIG. 1.—The developing social status during 200 intrastrain staged fights among the C albinos.

W_5 showed no signs of aggressiveness and was the only one of these mice which never contested the position of the mice with which he was matched. He was 4.5 gm. lighter than any of the other albinos at this period and gained no weight during the entire course of the experiments. He finally showed definite signs of weakness and eventually died. Autopsy revealed a greatly enlarged heart and marked peritoneal edema. The behavior of W_5 can, therefore, scarcely be considered as normal.

THE C-3H AGOUTI STRAIN

In our hands this stock seemed most lethargic in its general behavior. Any one of them might sit in the fighting cage with eyes almost closed, while it shook its head from side to side and paid no attention to its opponent. At times this behavior was so extreme as to suggest impaired vision. In the first encounters these mice were cautiously aggressive and indulged in feints rather than proceeding to actual skirmishes. The early fights were not so full of action or so long or frequent as were the fights between the albinos. It was not until after about 20 encounters that real battles became usual during the 20-minute combat periods. When they did fight, the agoutis either bit harder or were less able to defend themselves, for, in general, their fights resulted in more frequent injuries to one or both antagonists.

Throughout the series of 200 preliminary contact reactions Br_5 was either definitely first or was tied for that position. At the other extreme, Br_6 either occupied or was tied for the last place except once, early in the series, when it ranked next to last. In the final standing, after 200 contacts, Br_5 was slightly more successful than Br_4; and both of these were decidedly above Br_1, Br_2, and Br_6, which were judged to be approximately even in fighting prowess.

THE C-57 BLACK STRAIN

These mice were in better condition and were seemingly more vigorous than either of the preceding stocks. In general, they were more sensitive to handling and more restless. They were also more rapid in their movements. They sometimes showed less aggressiveness than either of the other two stocks; usually, however, they fought more readily, longer, and more rapidly than did either of the others. Prior to conditioning, no one of our black mice, with the possible exception of Bl_3, which had been severely defeated during Miss Fales's experiments, was defeated in actual combat by a mouse from either of the other strains. The fights among themselves were frequent and fierce from the very beginning of the testing series. No feinting was observed, and each of the 200 contacts staged in the present preliminary series resulted in one or more active attacks. The readiness to fight at the first opportunity was in marked contrast to the other stocks. With this general picture it is somewhat surprising that before conditioning in our hands Bl_3 submitted passively most of the time.

A linear order was established after 80 fights, which remained stable through the following 120 battles. It was as follows: Bl_2, Bl_5, Bl_1, Bl_4, Bl_3. There was an indication that the last three mice were less aggressive than the other two, which was apparent by the end of the first 10 fights. The only change that occurred after the first 50 combats was that Bl_2 replaced Bl_5 as the alpha-mouse. It is worthy of note that all the battles between these two aggressive black mice were fiercer and more intense than were any others in these testing series.

A close study of the behavior of Bl_2 and Bl_5 suggests that the latter may actually have been physically superior as a fighter. He was, however, perhaps more easily intimidated. The first 50 intrastrain combats between the blacks included 5 encounters between these two. The fights were always severe. Bl_2 tired more rapidly than its opponent. One or two added vigorous onslaughts by Bl_5 after Bl_2 was tired would usually suffice for a submission by the latter. In the meantime Bl_2 had been defeating all its other opponents and was becoming more and more aggressive. During the sixth round robin of intrastrain battles (there were 10 fights to a round robin) Bl_2 began to attack as soon as the partition was lifted. In previous pair-contacts there had been some time spent in mutual threats and observations. From that time on, Bl_2 consistently defeated Bl_5, which never attained this degree of aggressiveness. Some reversals of form were observed after short periods of isolation, which extended up to a week in length. In each of these instances Bl_2 did not immediately attack his opponent; on such occasions Bl_5 was the winner until Bl_2 again displayed his usual sharp aggressiveness.

With all three strains the order, once set up, remained relatively stable at both extremes but fluctuated somewhat in the mid-ranks. Unlike the albinos and the agoutis, the blacks soon established a linear order which was maintained. In fact, the differences between the subordinate black mice tended to become intensified as the fights proceeded. With the other two strains, and particularly with the agoutis, the differences in the lower

ranks tended to become less extreme as time went on. It appeared as though the agoutis particularly became more completely submissive to their dominants as a result of successive defeats; and, as they did so, they showed decreased hostility among themselves.

Effects of Conditioning

The influence of successive losses on subsequent fighting behavior

The C albinos—W_1 had been the alpha-mouse among the albinos for 140 battles when the conditioning experiments were begun; he, himself, had fought 28 times. There was no indication of a threatened shift in dominance; rather, his social status was becoming more firmly established. If this status could be changed following a series of defeats, we should have good evidence that the loss of dominance was a direct result of such experience.

There was a complicating factor which we attempted to overcome: isolation is important in determining fighting behavior. Mice which are regularly submissive often become aggressive if they have been kept isolated from their fellows for varying periods of time. Analogously, dominant animals are not quite so prone to attack a former subordinate after similar isolation. For this reason it is not entirely valid to assume that relative changes in status result from conditioning a particular mouse if the individual in question has had no contact with those against which he is finally tested during the conditioning period, provided that the period is relatively long (several weeks or more).

In order to circumvent the effects of isolation, W_1 was fought against Bl_2 twice a day for 8 days. Bl_2 almost invariably attacked its opponent as soon as the partition was lifted, and he showed signs of aggressiveness (tail-beating, etc.) whenever the partition was rattled. W_1 showed active resistance during the first 3 battles but was never able to hold out against Bl_2 for more than 15 seconds at most, despite the fact that both animals were of approximately equal weight and were each at the top of his own social order. After the twelfth defeat W_1 submitted passively as soon as Bl_2 attacked actively before the end of the exposure period, and the conditioning was thus regular and relatively severe. On the ninth day the albinos were fought among themselves in the manner described for the earlier intrastrain battles.

During the first round robin of intrastrain contacts W_1 submitted passively to every opponent, including even W_5, which had first submitted to W_1. None of these relatively passive albinos showed aggressiveness; hence the uniform submissiveness of W_1 was the more striking. Submissions were usually given only in the presence of an aggressive individual. Bl_3 and W_5 were exceptions to this general rule; they often submitted passively to the rattling of the partition or the proximity of the weighing box or even to a female shortly after her introduction into the cage. The conditioned submission of W_1 was similarly nondiscriminatory. These seem to be unoriented fear reactions rather than typical submission.

After two more series of pair-contacts—a total of 30 such encounters during the same day—W_1 was no longer completely submissive; he still showed no aggressiveness. On the following day he was threatened by W_2 with appropriate tail-beating and hair-raising and replied in kind. After 30 more passive but unsubmissive contacts W_2 again threatened, and he was attacked and promptly beaten by W_1. The battle was severe and lasted 50 seconds. After this fight the old order of dominance was quickly established.

At this point, W_1 was exposed to Bl_3, which, as usual, showed random, nonaggressive behavior. W_1 remained in its corner of the cage in a rigid attitude while Bl_3 explored the cage, and W_1 reared on its hind quarters as Bl_3 approached. Bl_3 also reared and squealed and went through all the signs of submission. During the remainder of the 20-minute period both mice explored the cage freely but reared on their haunches each time they approached each other. This suggested that W_1 might be reacting to Bl_3 as a member of the black strain. It was, therefore, placed with the nonaggressive agouti, Br_6, with which it had had no recent contacts. The same cautious pattern of behavior was shown by both animals. In the presence of the albinos, however, W_1 reassumed its old dominant attitude.

The foregoing behavior suggests that severe conditioning over a short period causes random submissive behavior, which can be interpreted as an unoriented fear reaction. After such conditioning, the mouse was able to readjust itself in a relatively short length of time and to assume its former dominance. If it had met with more active resistance from its own group during the first few postconditioning encounters, it would probably not have reassumed its aggressive attitude so quickly. The effect of conditioning W_2 in the same manner described for W_1 points to this conclusion. W_2 was attacked by W_1 during the early postconditioning intrastrain battles and retained its submissive attitude until after a series of passive encounters with the ultra-meek W_5, which covered a period of 10 days and began 2 weeks after the 8-day conditioning period.

If such an intermediate mouse does not meet any of its superiors after a series of defeats, it, too, is able to readjust fairly rapidly. Thus W_3 was given the usual series of 16 defeats by Bl_2 in 8 days and was then exposed only to the unaggressive W_5 until it resumed its normal behavior to the latter. W_3 was then able immediately to take its former position in the group despite threats from the alpha-mouse, W_1.

W_7 was the dominant animal in a group of four albino males of the following generation. It was treated to the customary series of 16 defeats in 8 days. The results paralleled those described for W_1 in most details. It took slightly longer for W_7 to reachieve its former position after the downward conditioning.

At this point an attempt was made to bring about a lasting change in the order of dominance among the oldest group of albinos. In order to avoid complications resulting from isolation from other members of the group, these mice were fought daily in round-robin fashion. In addition, the alpha-mouse, W_1, was made to meet several different black or agouti mice each day. The number of such pair-contacts varied from day to day. They were always continued until one or two severe defeats were suffered daily by W_1. Intrastrain contacts were arranged each morning; interstrain combats were staged in the afternoon.

Under this sytem of conditioning, the behavior of W_1 varied considerably. Most of the time he showed pronounced pacific tendencies in the presence of black or agouti animals; though he seldom attacked, he usually fought vigorously against an attacker. He seemed to show no discrimination between individual mice. After 2 weeks the other albinos, with the exception of W_5, which was ill, ceased to submit passively to W_1. At this time W_5 was replaced by $W_{5'}$, an albino from a different substrain. $W_{5'}$ had earlier been defeated 3 times in encounters with W_1, but now the entire social order was upset. $W_{5'}$ replaced W_1 as the dominant mouse, and the latter was now on a par with the others.

$W_{5'}$ was removed in a few days in order to simplify the situation, and the encounters

continued otherwise according to schedule. W_1 was still being defeated regularly by black or agouti mice and now submitted passively without showing resistance. W_3 became the dominant albino in this lot, and W_1 submitted passively to all his former subordinates. This conditioning series lasted for 35 days, during which time, W_1 had been defeated at least 47 times. In the 2 months that followed, W_1 did not regain his former status and showed no signs of aggressiveness. At first he submitted passively when in the presence of another mouse. He stopped doing so after a time but submitted immediately in the face of any slight show of aggressiveness by another mouse.

The summer quarter intervened. During this period of 4 months W_1 remained isolated. When he was again fought against the members of his group in the autumn, his former aggressiveness had been regained.

The C-3H agoutis.—Br_5, the most successful of the agouti strain, was given the standard series of defeats (16 severe losses in 8 days) and then fought again against its fellows. Br_4 became dominant; it had already been a close second, and Br_5 did not recover its former status until Br_4 was experimentally lowered in the scale.

Br_4, the new dominant, was consistently beaten in contacts with the blacks without interrupting its daily exposure to the other members of its group of agoutis. At the end of 3 weeks of such treatment he was submitting passively to all except the nonaggressive Br_6, which usually submitted faster than he could. Br_5 became dominant once more, and the order remained stable during 10 more days of experimentation.

As with the albinos, an attempt was made to repeat the results with agoutis of the succeeding generation. The outcome was obscured because Br_8, which had previously been judged to be unaggressive, suddenly changed its behavior and dominated the entire group, including Br_7, the former dominant, which was in process of being conditioned downward. The increased meekness of Br_7 after partial conditioning may have stimulated Br_8 to display his aggressiveness. Uhrich's (1938) observations indicate that reversals do occur at times in such groups of mice without known causation; hence, in the absence of more critical evidence the suggested explanation remains a guess.

The C-57 blacks.—The ordinary procedure was modified somewhat because none of our available mice were capable of administering defeats to the dominant blacks. For this reason, Bl_5, which was in beta-position in the social order of the blacks, was fought against the dominant Bl_2, once daily for the first 4 days. It was quite an effort for Bl_2 to defeat Bl_5 during their initial battles. After the defeats became more routine, the two were matched twice daily. After the fifth day, intrastrain fights were also staged daily between all these blacks with the exception of the omega-individual, Bl_3; and the formerly aggressive Bl_5 went to the bottom of the group with which it was being fought in 15 days of such treatment.

Less direct evidence of the effect produced by defeats is furnished by our experience with Bl_2, the most aggressive mouse in our entire colony to date. Despite some relatively serious injuries and a rigorous fighting regime, Bl_2 could always be counted on to attack an opponent fiercely the moment the partition was raised in the fighting cage. If the tip of an opponent's tail chanced to protrude beneath the partition, Bl_2 would attack that. In nearly 200 battles, Bl_2 lost only some 10 times; he was always less prone to attack after having been defeated. Bl_5 showed similar tendencies.

Interstrain tests.—Before conditioning, Bl_5 invariably fought all the males of the C-3H and C albino strains and was victorious in all encounters except one, which was accidentally disturbed. After conditioning he was submissive in the presence of W_3, W_6, Br_5, and

Br_2 and pacific in the presence of W_1, W_2, and Br_6. Br_2 was the only mouse which made an active attack in these postconditioning, interstrain contacts, and Bl_5 submitted passively both before and after the attack.

The effect of successive defeats cannot be ascribed to fatigue or injury. When at all able to do so, Bl_2 was normally fighting from 4 to 6 times daily during this period in addition to intrastrain battles and had, moreover, been injured on several occasions. Three of its injuries necessitated several days of complete inactivity. None of the mice which were showing the effects of defeat were forced through so rigorous a schedule, nor did any of them sustain appreciable, inactivating injuries.

A victorious, favorably conditioned animal will fight successfully against heavy odds, while an oft defeated one of similar constitution and stamina is psychically handicapped to such an extent that it seldom does well even under favorable conditions. Psychic, rather than physical, factors seem most important here.

THE INFLUENCE OF SUCCESSIVE VICTORIES ON SUBSEQUENT FIGHTING BEHAVIOR

The albinos.—The most effective way of testing the effect of successive victories would be to see whether a change in status could be brought about in one of the completely subordinate animals and, if so, how great a change was possible. The difficulty in such experimentation was that, for the most part, such animals would not fight or even threaten an opponent. In an attempt to surmount this difficulty, W_5, the passive omega-mouse of its lot, was placed in a neutral cage with W_6 for several hours daily. W_6 was nonaggressive, and W_5 soon stopped submitting to it. At this point W_5 died.

W_{15} was the omega-mouse in a group of five second-generation albinos bred from our original stock. As usual with these second-generation mice, all those grouped together were litter mates. W_{12} was second lowest, and neither animal was aggressive. They were caged together for several hours daily, as has just been described, with no apparent change in behavior toward each other. At the end of a week W_{15} was mated. The next encounter between the two males came just after an observed copulation. W_{12} pursued W_{15} all around the fighting cage and sniffed insistently at his hind quarters. W_{12} finally nipped at W_{15}, which promptly submitted. At this point, W_{13}, which was approximately on a social par with W_{12}, was introduced. It also pursued W_{15}, which submitted. W_{12} tried to crowd the newcomer away, and a fight ensued. The entire group was matched in round-robin fashion with the exception of W_{12}. W_{13} was beaten in a routine fight and was rematched immediately with W_{12}, which attacked after W_{13} had first submitted passively.

On subsequent days this practice was continued. W_{12} was omitted from the intrastrain battles but was matched with a mouse of its group just after the latter had been beaten. After a week and a half of such a "build-up" W_{12} became quite aggressive and defeated W_{11} (on a par with W_{13}), W_{15}, and W_{13} regularly, regardless of whether they had been beaten previously. At the end of 18 days of such treatment W_{12} defeated the dominant mouse of its group (W_{14}), from which it had been isolated for the entire period. It must be noted that W_{14}, for all its dominating position, was not generally aggressive. It dominated by threats after a very few battles. The fight with W_{12} was the first severe combat in its history. Even so, the results show that the aggressiveness of W_{12} was strongly influenced by its run of successful fighting.

The C-3H agoutis.—Our even more striking experience with conditioning Br_6, the least aggressive agouti, so that he became a vigorously aggressive individual, has been

told elsewhere (Allee, 1942*a*, 1942*b*). He not only became the dominant mouse in his own group as a result of some 6 weeks of careful social manipulation, but he also became aggressive enough to give Bl_2 a long hard fight—so hard, in fact, that Br_6 died as a result.

The C-57 blacks.—All of the techniques which were used with the albinos and agoutis were tried with Bl_3, which was the least aggressive of these blacks. This animal was sensitive to handling and did not fight under any of the conditions mentioned. Finally, he was isolated for 5 weeks. His first encounter after isolation was with Bl_4, which had formerly ranked just above him. Both moved about the cage for several minutes: Bl_3 then sniffed at his relatively mild opponent, which rose on his haunches and squealed. Both mice explored opposite corners of the cage for awhile. Then they touched vibrissae; and Bl_4 squealed, ran, and reared up in a corner. Bl_3 approached and groomed it for several seconds. Again Bl_4 ran, squealed, and reared up as before. Bl_3 followed cautiously, waited until Bl_4's back was turned, and attacked. Submission was prompt; Bl_3 attacked once more before the partition was interposed between them.

Bl_3 then attacked and defeated Bl_1 when the two were matched after the latter had recently been beaten by the dominant Bl_2. In the meantime, Bl_2 suffered one of its rare defeats by Bl_5, and Bl_3 was matched with him. There were 3 severe fights in which both mice attacked practically simultaneously. These fights were stopped before a decision was reached, in order to shield Bl_3 from possible early defeat. Later in the day Bl_3 defeated Bl_5 in a single severe battle.

A week later the battles were repeated, with the same results. Then the animals were fought daily, and Bl_3 again went to his earlier place at the bottom of the social order after 4 more days of fighting. He was then isolated for 8 weeks and was again gradually "built up" by being matched with low-ranking agoutis, a method which had proved effective with Br_6.

After only 3 days of such opposition he again became obviously aggressive. He was matched against each of the three bottom blacks of his group and beat them all. At the end of the week, Bl_3 was again put in with Bl_5, which had just been beaten by his superior, Bl_2, and won. The interval between the defeat of Bl_5 and the latter's being matched with Bl_3 was gradually increased to 3 hours, and Bl_3 still won. Bl_3 was then matched with the potent Bl_2 approximately 15 minutes after the latter had had a winning fight with Bl_5. Bl_3 was fresh and won. Two days later Bl_3, which had fought and won from all the blacks in the meantime except Bl_2, was again matched with this hard-fighting mouse. Both were fresh, and there was a real fight. Bl_3 had become extremely aggressive and defeated Bl_2 in a series of pitched battles.

The next series was fought 2 days later. It involved 30 minutes of intermittent fighting between Bl_3 and Bl_2. Both animals appeared to be completely exhausted several times during the encounters. Bl_2 finally won a complete victory. Bl_3 was passively submissive to any mouse of any strain with which he was matched from that time to the end of the experimental period 16 days later. At the time of his winning the dominant position, Bl_3 was the heaviest mouse in its group by a slight margin.

Isolation, or some other device to overcome the reluctance of low-ranking animals to fight, appears to be a necessary prerequisite for their upward conditioning. The normal reluctance may be partly a matter of memory and of fear, both produced by previous defeats. It is far easier to reduce the aggressiveness of a superior animal by a series of defeats than it is to get an animal at the bottom of his social order to show signs of aggressiveness. The latter process is slow and painstaking, and the experimenter must be

continually alert and must modify his procedure to meet suggestions given by the slight behavior clues which indicate the degree of progress his subject is making.

Discussion

It is difficult to summarize adequately such complex behavior patterns as those which we have been considering. The different categories shade off from their modes. There are exceptions to be evaluated and individual variations of predominant patterns to be considered, even when they are not described in the final account. Probably no one except the observer himself could get a clear picture or even an approximately accurate impression of the whole matter at hand, even though the account were to be spun out far beyond publishable length. Such troubles increase when attempts are made to tabulate observations in definite classes. We hope that readers will appreciate our difficulties. We have tried to be conservative in our statements, and we have seen many fights, the results of which are barely suggested in the foregoing pages. Despite this extensive experience, we feel that we have merely made a modest sampling of the fighting behavior of these mice, not that we have reached definitive conclusions.

This study is one of an informal series, with which the second author is concerned, in which an analysis is being made of factors which make for social dominance as contrasted with social subordination. Without going into details, which would differ with the animals studied, we know that the following factors are involved, at least in many cases:

1. *Physiological state.*—This factor includes such matters as fatigue, which has been seen to be effective in some of the fights among these mice—so much so that when we were trying to condition a mouse to be more aggressive it was standard practice to pit it against another that had just been in a hard fight. Illness also tended to reduce aggressiveness, as did severe injuries with most mice.

Under the conditions of these experiments starvation for 12-, 24-, and 36-hour periods made no difference in aggressiveness. Only once was there a fight over food, and that did not occur during or soon after a starvation test. Witholding water for 6 or 8 hours from isolated or communally penned mice and then introducing a water bottle with a small nozzle, so that only one mouse could drink at a time, did not stimulate fighting.

Temperature is another easily controlled factor which affects the physiological state of mice as well as other animals. We found that fights were possible over a temperature range from 18° to 28° C. The combats were more sluggish and shorter at the higher temperatures; and inexperienced, unaggressive mice did not fight at 27°–28° C. Wider variations in range were not tried. There was no differential response to temperature on the part of the various strains that was detected by our methods.

The action of various hormones may also affect the physiological state and so have a controlling action on aggressive behavior. This had been demonstrated for the androgen, testosterone propionate, with a number of different kinds of animals (cf. Allee, Collias, and Lutherman, 1939). Such hormones supposedly affect aggressiveness by acting through the nervous system. These strains of mice which differ in innate aggressiveness appear to offer promising material for hormonal experimentation.

2. *Weight.*—Uhrich (1940) reported suggestive evidence that differences in weight tended to make an effective difference in the winning of fights. The heavier mouse won, in his series, 6 times and lost 1 time when weight differences were as much as 5 gm. (approximately 15–20 per cent).

In our studies we have comparable data regarding the relation between weight and success in preconditioned, intrastrain fights for 53 mice. These included 22 blacks, 12 agoutis, and 19 albino mice. The departure of weight from the mean for the strain was as much as −5 gm. with one mouse and −4 gm. with another; the others all ranged between −3 and +3 gm. from the average for the given strain. The coefficient of correlation between success and weight in these cases is positive and slight ($r=0.2852\pm0.1356$ S.E.); however tested statistically, this degree of correlation is just on the border line of validity. A larger series of animals is needed for a conclusive test.

3. *Heredity.*—There seems little room for doubt that some of the factors which make for dominance can be inherited. The greater power to win fights by the black strain, as contrasted particularly with the more passive albinos, makes this point. The distinction between either of these and the intermediate agouti strain is also clear-cut. The strain differences which determine success in fighting have not been separated either genetically or, for that matter, by controlled, analytical experimentation. Several factors can be recognized which contribute to success. In addition to possible hereditary differences in physiological state and weight, there are also aggressiveness, ability in the attack, ability in the defense, and general stamina, which are easily seen to be important.

Aggressiveness toward the keeper differs from aggressiveness toward other mice. All three people who have cared for this colony—Miss Fales, Mr. Ginsburg, and now Miss Elizabeth Beeman—have been bitten by the albinos. The blacks are most pacific, according to this criterion; and the agoutis are intermediate. Aggressiveness in mice is a complex quality which needs further careful analysis.

The albinos tended to stop and explore the fighting cage even in the presence of another mouse. To some extent the agoutis showed the same behavior, while, in contrast to both, the blacks usually attacked. Before being conditioned, they had a strong tendency to attack even if low in the social scale and if easily beaten.

In view of the varying fortunes in the series of fights between Bl_2 and Bl_5, which have been given in a previous section and which finally resulted in the emergence of Bl_2 as the dominant animal of our whole colony, it cannot be unequivocally stated that the physically superior mouse becomes the dominant one. If the animals are normal and of comparable weights, even when unconditioned, natural dominance depends on a number of traits. One of these is aggressiveness.

We are not prepared to evaluate fully the role of aggressive behavior in the winning of fights. Its importance as a contributing factor is readily apparent. Aggressiveness, apparently, is an inherited trait as far as initial contacts go. It is, however, subject to great modification over varying periods of time, after conditioning. In general, our evidence shows that unconditioned fighting ability is a complex strain character not necessarily determined by any single gene (since the strain differences are largely multiple-factor differences) and subject to great environmental modification.

It has previously been mentioned that the agoutis sometimes showed more aggressiveness in initial encounters than did the blacks, but the latter invariably won such pair-contacts. This does not refute the importance of the role played by general aggressive behavior, for two reasons: (1) Decisive initial encounters are usually settled through actual battles if the animals are exposed to each other for a sufficient length of time. Submissive responses to aggressiveness do not usually occur until after several defeats, and this is an indication that learning is an important factor in such submissions. (2) The aggressiveness displayed by the agoutis before initial fights was confined to hair-

fluffing and tail-beating. The aggressiveness displayed by superior mice after fighting experience consists of a direct and vigorous attack, the results of which, through repeated experiences, may well come to be associated with pain and defeat by the inferior mice. This is the combination which presumably determines the social order in nature, if, in fact, it exists there among populations of mice.

Elton (1942, p. 205) comments on fatal attacks in small caged colonies of voles (*Microtus*) and states: "This social antagonism among voles probably exists in nature, and may be very important at different levels of population, and may prove to explain some of the mortality at higher numbers."

There are other phases of behavior—nest-building proclivities, for example—which differ in the different strains we have studied. This is not the place to discuss further the relations between heredity and behavior in these mice; nor, indeed, is this our problem. We have primarily used the hereditary differences as originally discovered by others as a tool with which to find evidence of the effect of conditioning on dominance-subordination patterns of behavior in these mice.

4. *Conditioning.*—It is somewhat surprising how rapidly the effect of previous failure in fighting can be detected in the fighting behavior of a given mouse. This is more noticeably true with mice which are intermediate in the social order of their group. Such mice are already partly conditioned to win and partly conditioned to lose by their routine contacts; hence it is easier to cause an intermediate mouse to lose status than it is to do the same with a dominant individual, and, on the other hand, it is easier to condition an intermediate mouse so that he advances in his social scale than one which stands at or near the bottom of his own group.

As yet, we have only a small number of cases in which mice have been conditioned to become more aggressive in behavior and more successful in fighting. We are impressed by the results obtained, because the mice which were lowest in the social scale often showed extreme subordination. Their responses were unorganized in the fighting cage and suggested strongly behavior disintegration similar to that described for rats (Anderson, 1941; Hall, 1941). Hence we find the conditioning upward of such mice as Bl_3, for example, particularly impressive and convincing.

Another effect of conditioning is worthy of note. The correlation between aggressiveness, as shown by starting a fight, and success in fighting, becomes much higher as a result of conditioning.

As with many other biological processes, including other phases of conditioning, the retention of the effects of training depends in part on its duration. This was best shown by our experience with the effects of continued defeats. If such conditioning was freed from the counteracting effects of isolation by frequent contacts within the group, then long-term treatment had relatively lasting effects. Severe conditioning for a short period altered the behavior of a dominant animal for a relatively short time only. Such short-time conditioning treatment had a more lasting effect on an intermediate animal, since it was really extended, in such an instance, through defeats administered by superior animals in its own group after the experimental conditioning was over. Where this factor was experimentally controlled, an intermediate mouse recovered from the effects of a short series of defeats as rapidly as did a dominant individual.

Conditioning can also be accomplished by rearing young mice together in the same cage. For controls, one contrasts their fighting behavior with that of mice which have been reared apart. Our experience on this point is not extensive, but we do have indications with the albino and the black strains that the fighting tendencies in such commu-

nally reared mice are less than if they were reared, from weaning on, in isolation. These indications hold even in the more provocative situations in which fertile females are present.

Two other factors which have been reported to affect social dominance are: (1) what may be called the "home-cage effect," which is, at least in a general way, an aspect of territoriality, and (2) age. We did not find that limited age differences were important when they were divorced from differences in weight of over 5 gm. and also from differences in experience in fighting. Our tests did not include differences of more than 3 or 4 months. In the strains which were regularly tested either in intrastrain or in interstrain pair-contacts, the mice were all nearly of the same age; hence, for the purposes of these experiments this factor was effectively eliminated. The possibility of a home-cage effect was also eliminated, since the contests were staged on neutral ground except in those cases in which, during a conditioning series, we were attempting to favor one of the mice. In our hands and with these mice, we did not find any strong evidence that the home cage exerted great influence on the outcome of a fight unless the home cage contained a female. The presence of such a female, then, becomes the last of the factors that make for dominance with which we have worked in these experiments.

The behavior of these animals before and after conditioning suggests a certain analogy to the conditioned reflex in Pavlov's sense (1927, p. 26), though a more complex category of nervous activity is being dealt with. In Pavlov's words:

> The fundamental requisite [to establish a conditioned reflex] is that any external stimulus which is to become the signal in a conditioned reflex must overlap in point of time with the action of an unconditioned stimulus. . . . . It is not enough that there should be over-lapping between the two stimuli, it is also and equally necessary that the conditioned stimulus should begin to operate before the unconditioned stimulus comes into action.

The most generalized unconditioned stimulus for fighting in an aggressive male mouse is the proximity of another male mouse. Aggressive deportment before a battle, if it comes to be associated with defeat, soon elicits the submissive response, even if no battle has taken place. Likewise, the transfer to the fighting cage before the battle also provokes a certain type of fighting behavior, depending on the previous experience of the animal in the fighting cage. If the mice are allowed to remain in the fighting cage under the experimental conditions centering about the battle (i.e., raised partition, top lighting, and the presence of another male mouse) and if they remain pacific during several such encounters, it soon becomes difficult to elicit the fighting response from many of them. The fighting cage has lost its efficacy as a conditioned stimulus for fighting, and the animal responds with a random behavior pattern, or even by the social action of grooming its "opponent." For these reasons, care must be taken to insure active battles for all mice that are to respond belligerently under the experimental conditions, even if short periods of inactivity must be interpolated to accomplish this. In the absence of such precautions the number of active battles is greatly diminished.

Perhaps the analogy to the reflex will help explain several obscure points having to do with fighting behavior under the conditions described. Ordinarily, two animals are promptly separated by the partition after one of them has submitted, and no further fighting can then take place. Infrequently, an intermediate animal will turn on a "superior" adversary directly after having been defeated, and the "victor" may than submit without a battle. During the course of these experiments this was never observed when the victor was a very successful animal at the top of its own order, but it was observed in several instances involving battles between intermediate mice when the par-

tition was not interposed promptly between the contenders. If it is true, as these and other experiments indicate, that mice respond to deportment rather than to individuality as such, it may well be that the foregoing can be accounted for as follows:

The less successful animal submits, either after an actual battle or after exposure to the aggressive behavior which it has come to associate with defeat and which may be loosely analogized to a conditioned stimulus. After submission the victor retires to an unoccupied portion of the cage and ceases to show signs of aggressiveness, having been partly conditioned to this type of behavior by the fact that the animals are normally separated directly after submission. This may provide a weakly conditioned stimulus for the mouse that has just submitted to attack an unaggressive victor, if the former was not too decisively beaten in the previous encounter. This hypothesis has not been tested experimentally, and it is difficult to push the analysis further until more data make it possible to subdivide fighting behavior into various aspects, especially with respect to the following: (1) the effects of handling, (2) prefighting aggressiveness, (3) initiating a fight, (4) the fight proper, (5) submission, and (6) behavior after submission.

In the absence of a more adequately diagnostic term, we propose to refer to the type of behavior and behavior changes that we were able to induce as a "conditioned automatism."

Finally, we need to emphasize that within each strain the mice show decided individuality as regards both aggressiveness and fighting techniques. Some of the individual differences we can trace back to previous conditioning; others we cannot. While evidence on this point is far from complete, there are strong indications that the young mice reared in our laboratory showed individual variations which resulted from differences which the animals possessed at birth. Such differences may, or may not, be truly genetic. We remember that the Dionne quintuplets (Blatz, Millichamp, and Charles, 1937) showed clear evidence of differing among themselves in social status. Presumably, these were an identical set of sisters, each with the same heredity as the others. Their differences may well have arisen from their relations in and to their intrauterine environment. Such differences would also be expected among mice. The extent and importance of such fetal interrelationships and particularly their bearing on adult behavior, especially on social behavior, is an intriguing problem.

With mice which are not litter mates, even with uniform handling, controlled external conditions, and close inbreeding, there are a number of subtle factors which may act to produce individual differences in behavior.

In the guinea pig, Wright (1926) has shown that, though the white spotting pattern is determined largely by one pair of alleles, the age of the mother is an important factor in establishing the extent of the spotted areas. The data of Castle (1936*a* and *b*) have demonstrated a decided effect of the size of the mother on birth weight and litter size. Bittner (1937, 1939*a*) has shown that cancerous strains of mice will transmit a tendency to form mammary cancers to noncancerous strains which they foster. This tendency is probably resident in the mother's milk. Wright (1934) has demonstrated the best-known case for a strict maternal-age effect acting independently of the known gene constitution of an animal. His data demonstrate conclusively that age of the mother, per se, is responsible for the relative changes in frequency of the numbers of digits produced in genetically uniform material.

Thurstone and Jenkins (1931) have made a study of such factors as are expressed in human material. Their data include ten thousand cases, and they seem to have avoided most of the common pitfalls that usually attend such investigations. They found

a negative correlation between growth and birth order; a significantly high proportion of cases of pyloric stenosis, tuberculosis, criminality, epilepsy, schizophrenia, feeble-mindedness, albinism, congenital cataract, and genius among the first-born. Mongolism was most frequent among the last-born. There was a general progressive increase of I.Q. from the first- to the eighth-born. The age of the parents did not prove to be a factor in these I.Q. studies.

In general, it should be emphasized that maternal and other extra-genetic influences on genetic and seeming genetic characters are manifold and well established; and, though it seems logical to refer unconditioned behavior traits to genetic differences, it is impossible to postulate the nature of these differences without resorting to a rigorous and exacting demonstration of any alleged premises.

Summary

1. Definite genetic behavior differences exist between highly inbred strains of mice, some of which are consistently demonstrable under specified conditions. In the strains studied, the C-57 blacks were superior, the C-3H agoutis were intermediate, and the C albinos were least effective, as judged by the outcome of actual battles. Other consistent differences were also noted.

2. Superiority in fighting appears to result from a multiplicity of psychic and physical factors. The mice in a small group show a social organization which is based on dominance-subordination relations, determined in the last analysis by superiority in winning fights.

3. The order of dominance in both isolated and communally penned mice varies from group to group and has temporary stability. It is most stable at the extremes. Relatively stable social structures may appear after many battles.

4. Individual mice may be conditioned to be less aggressive as a result of repeated defeats. They can also be made more aggressive as a result of continued victories over submissive mice. Both types of conditioning strongly affect the ability of a given mouse to secure or to maintain high social status.

5. It is far easier to condition a socially superior mouse downward in the social scale by a series of defeats than it is to condition a socially inferior mouse upward. Individuals which are intermediate in their social position are most readily conditioned in either direction.

6. Factors known to be of importance in determining social dominance include: physiological state; differences in weight, if over 5 gm. (15–20 per cent); hereditary differences in aggressiveness and fighting ability; and conditioning.

7. The presence of a female not only stimulates fighting among otherwise passive male mice, but she also tends to stimulate her mate to make a successful defense of his home cage or of any other cage into which the pair was introduced prior to the entry of an intruder.

8. Because of the small number of animals worked with (some forty-five were studied intensively) and because of the standardization of procedures, no finality is claimed for the results which have been presented. Comparison with other work suggests a certain amount of universality.

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